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Novel role for human beta defensin-2 as a protease inhibitor In protecting HaCaT cells monolayer against *S. aureus* Secreted proteases.

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Abstract:

Novel role for human beta defensin-2 as a protease inhibitor in protecting keratinocytes against *Staphylococcus aureus* secreted proteases.

Atopic dermatitis (AD) patients' exhibit increased susceptibility to viral and microbial infections, especially *Staphylococcus aureus (S. aureus)*. Evidence suggests that *S. aureus* secreted proteases are responsible for increased breakdown of junctional and desmosomal adhesions, that help to maintain skin barrier function. I therefore examined the effect of *S. aureus* secreted exotoxins (proteases), on an intact monolayer of HaCaT cells, a keratinocyte cell line. Damage to the HaCaT monolayer was observed as a loss in tight junction and desmosomal contacts, which were visualized throughout the monolayer when I stained them (after staining) with specific antibodies. I quantitated those effects by Western blotting and by measuring transepidermal electrical resistance (TEER).

S. aureus secreted serine protease (V8) was the key agent responsible for the gross effects on HaCaT monolayers. I also discovered that pre-treatment of HaCaTs cells with both IL-1β and LTA protected monolayer from *S. aureus* protease and this could be attributed to increase secretion of a novel peptide. I further demonstrate that hBD2 a novel peptide act as an ant-protease that inhibit both V8 & staphopain B. Importantly, novel peptide could reduce the deleterious effect of *S. aureus* conditioned media, which contain higher concentration of bothV8 and staphopain B proteases. For further specification I compared *S.aureus* parent strain 8325-4 and their mutant strains L17 (SspB), L22 (SspA) and (L27) Scpa and SH1000. *S. aureus* proteases effects were observed in the presence or absence of recombinant, synthetic and linear scrambled novel peptides. The protective role played by skin commensal was tested by heat killing *S. Epidermidis*. I found HaCaT monolayer was protected from *S. aureus* secreted proteases when pretreated with heat killed *S. Epidermidis*. Thus, I concluded that V8 protease may be the most important factor involved in breaking skin barrier of atopic dermatitis patients.

DECLARATION

There is no any part of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or another institute of learning

Dr AYUB AHMAD QURESHI

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LIST OF ABBREVIATIONS

AD	**Atopic dermatitis**
AJ	**Adherent Junction**
BCA	**Bicinchoninic Acid**
BSA	**Bovine Serum Albumin**
CE	**Cornified Cell Envelopes**
CLDN	**Claudins**
DAPI	**4,6- Diamidino-2-phenylindol**
DMSO	**Dimethyl Sulphoxide**
DS	**Desmosomes**
EGF	**Epithelial growth fator**
FITCI-Alb	**Fluorescein Isothiocyanate- Albumin**
HaCaT	**Human Keratinocyte Cell Line**
HKSE	**Heat killed *Staphylococcus.Epidermidis***
HRP	**Horseradish Peroxidase**
IL-1β	**Interleukin-1β**
KLK	**Kallikrein-related epidermal serine proteases**
LTA	**Lipoteichoic acid**
PBS	**Phosphate-Buffered Saline**
PFA	**Paraformaldehyde**
PGN	**Peptidoglycan**

PKC	**Protein kinase c**
QrtPCR	**Quantitative Reverse Transcription polymerase chain reaction**
S. aureus	***Staphylococcus aureus***
SB	**Stratum Basale**
SC	**Stratum Corneum**
SDS-PAGE	**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis**
SG	**Stratum Granulosum**
SL	**Stratum Lucidum**
SS	**Stratum Spinosum**
TGF	**Transforming growth factor**
TBS	**Tris-buffered Saline Solution**
TEER	**Trans Epithelial Electrical Resistance**
TEWL	**Trans Epidermal Water Loss**
TJs	**Tight Junctions**
UVR	**Ultraviolet Radiation**
UVA	**Ultraviolet Radiation A**
UVB	**Ultraviolet Radiation B**
VEGF	**Vascular endothelial growth factor**
ZO	**Zonula Occludens**

Chapter 1:

Structural and functional importance of skin barrier affected by *S.aureus* proteases in AD

1 Introduction 1.1 Human skin

Human skin is the outer covering of the body **(Pereira *et al.*, 2013).** The average adult human skin has a surface area of 1.5 to 2 m^2. There are two main kinds of human skin, Glabrous (non-hairy) and non-Glabrous (hair bearing). Glabrous skin is found on the palms and soles. Non-Glabrous skin has both hair follicles and sebaceous glands with wide variation between different body sites **(Howard *et al.*, 2010).**

1.1.1 Anatomical structures of the skin

Epidermis, dermis and hypodermis are three layers of the skin. Each layer differs in thickness, function and mechanical strength. Skin appendages include sweat glands, hair follicles, sebaceous gland and nails **(Fig 1.1)** (**In RoB et al., 2005)**.

The hypodermis is the innermost layer of the skin, and consists of a network of adipocytes (fatty tissue) and fibroblast (collagen secreting cells). The hypodermis is also known as the subcutaneous layer, and functions as both an insulator, conserving the body's heat, and as a shock absorber, protecting the inner organs. It also stores fat as an energy reserve for the body. The blood vessels, nerves, lymph vessels, and hair follicles also cross through this layer.

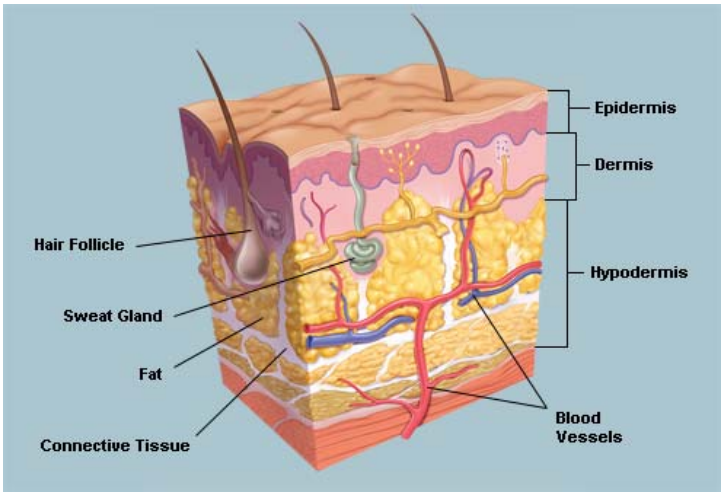


Fig- 1.1 the structure of human skin

Skin is composed of three layers: the epidermis, dermis and hypodermis. Adnexal structures of the skin include, hair follicles, glands associated with muscles, nerves and blood vessels (Image is taken from **WebMD 2009** with open permission)

The dermis

Dermis is made up of two sub layers; the papillary and reticular layers. The papillary dermis comprised of mostly areolar tissue and 20 % of the dermis, and the reticular dermis contains thick connective tissue and constitute 80% of the dermis. Dermis also contains most of the skin's specialized cells (fibroblast, macrophages, and adipocytes) and structures, which includes blood vessels **(Brown and Burn, 1996).** The "collagen" and "elastin" are fiber protein produced by dermal fibroblasts which provide strength and elasticity to the skin. The dermis contains appendages such as sweat glands, and hair follicles which are linked to the epidermis but project deep into the dermis. (**Naylor *et al.*, 2011).**

The epidermis

Epidermis is the outermost layer of the skin; it protects the body by acting as a frontline barrier against the environment **(Elias PM *et al*., 2008).** The epidermal barrier is continuously renewed by terminally differentiating keratinocytes **(Candi E *et al*., 2005).** Epidermal thickness varies in different types of skin, being thinnest on the eyelids and thickest on the palms and the soles of the feet. In addition to its main cellular components keratinocytes, the epidermis contains the following cell types; melanocytes (pigment producing cells), the Langerhans cells (antigen presenting cells involved in the immune system of the skin), Merkel cells and sensory nerves **(Naylor *et al.*, 2011).** The skin epidermis and dermis contains an easily accessible, immune privileged reservoir of adult stem cells for cell-based therapy **(Shi *et al.*, 2006).** The process of terminal differentiation

leads to the production of multilayers, stratified epithelium. The epidermis is made up of five sub-layers which are as follows, stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG), stratum lucidum (SL), stratum corneum (SC). Epidermal layers works together continually to rebuild the surface of the skin **(Marks and James G, 2006).**

Epidermal sub-layers

The stratum basale (SB) also known as stratum germinatum is the innermost layer of the epidermis, containing large columnar cells attached to the epidermal basement membrane at the dermo-epidermal junctions. The basal cell layer also contains melanocytes which produce coloring pigment (melanin). Basal cells proliferate and provide daughter cells which undergo terminal differentiation to produce other layers of the epidermis. The basal cells constantly migrate upward and differentiate into different cells in the spinous layer, which is the thickest layer of the epidermis and is involved in the synthesis of structural proteins. The structural proteins keratin five and fourteen which are present in the basal cell layer are replaced with keratin one and ten in the spinous layer. The spinous layer cells show spiny projections and are held together by desmosomal interaction. Upper spinous layer has lamellar bodies (LB), which contain ceramides cholesterol and free fatty acids. This layer also contains Langerhans cells. The cells of the spinous layer constantly move up to the stratum granulosum which is called the granular layer. Keratinocytes of the granular layer has keratohyline granules (KHG) which contains precursors of the cornified envelope (C.E) such as profilaggrin and loricrin **(Candi, E., et al. 2005).** As the cells move further towards the surface of the skin they get bigger and flatter and adhere together, then eventually become dehydrated and

die **(Figure 1.2) (Brown and Burns, 1996) (Marks and James G, 2006).** The dead skin cells make a thin and clear layer called stratum lucidum (SL), which appears translucent under a microscope. The stratum lucidum is located between the stratum granulosum and stratum corneum layers and is composed of three to five layers of dead flattened keratinocytes. The keratinocytes of the stratum lucidum do not have distinct boundaries and are filled with eleidin, an intermediate form of keratin.

The Lamellar bodies (LB) release their content into the intercellular space of the stratum corneum (SC). The content of the KHG and LB content covalently cross-linked with transglytaminase-1 form C.E **(Cabral. A et al, 2001).**

This arrangement of terminally differentiated cells with lipid matrix provides skin its permeability barrier which is constantly renewing throughout the life. In a healthy skin this process of terminal differentiation is highly regulated and make a constant balance between desquamation **(**sloughing off the dead cells) of the terminal layer and proliferation in the basal layer **(Liu *et al*., 2003).**

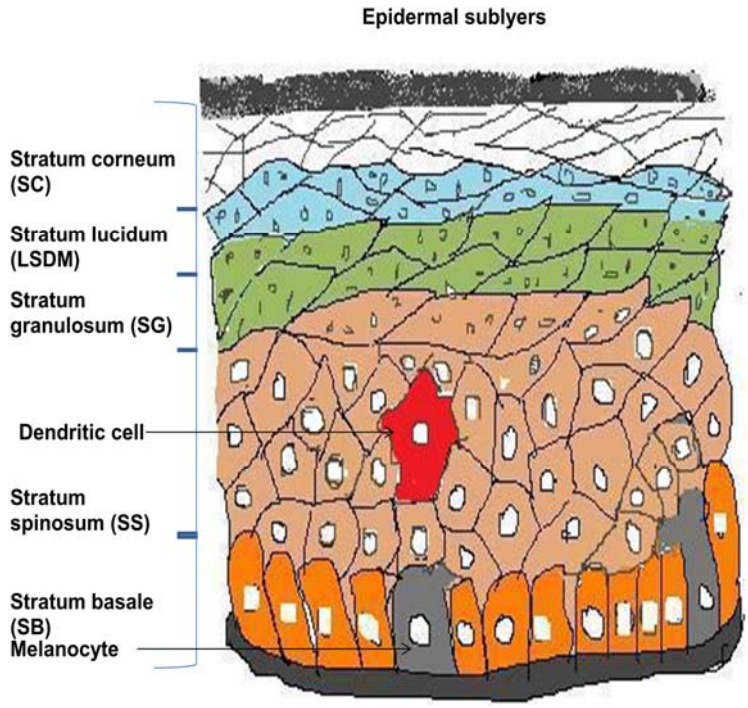


Figure 1.2 Schematic representations of epidermal sub-layers

The epidermis consists of five layers stratum basale which has melanocytes; the stratum spinosum which has Langerhans cell; the stratum granulosum; the stratum lucidum and the stratum corneum. (Draw by Ayub Qureshi).

1.2 Skin Barrier

Normal skin barrier is formed by the contribution of cornified envelop, lipid matrix, corneodesmosomes, which are the desmosomal adhesions between corneocytes and tight junctions **(Ishida-Yamamato *et al.,* 2005) (Elias, P. M et al., 2008).**

1.2.1 Cornified envelope (CE) The CE is formed when flattened keratinocytes break down their plasma membrane and replace it with the insoluble proteins loricrin, involucrin, filaggrin, and small proline-rich proteins. These proteins are cross-linked together by the action of transglutaminases **(Eckert RL *et al.,* 2005).** The CE gives strength to the corneocytes and provides a scaffold for the binding of lipids, including ceramides which are present in the lamellae of the stratum corneum. Cells of the stratum granulosum secrete their contents into the extracellular space to make a lamellar matrix, composed of ceramides, cholesterol, cholesterol esters and fatty acids which retain and entrap water **(Feingold *et al.,* 2014).** Corneocytes of the cornified envelope (CE) are the "bricks" while lipid matrix is the "mortar" in the brick and mortar model of skin barrier integrity **(Nemes Z *et al.,* 1999).** If any component of this complex is damaged it can result in a disturbed barrier function **(Ohnemus U *et al.,* 2008).** Filaggrin (FLG) is expressed in the SG layer as a precursor protein called profilaggrin, which is dephosphorylated and cleaved by proteases into FLG monomers. The FLG monomers are further degraded to make natural moisturizing factors which maintain hydration of the upper SC **(Sandilands *et al.*, 2009).** The strong association of FLG mutation with AD is one of the most important genotype and phenotype linkage observed in human complex genetic disorders **(vanden Oord and Sheikh, 2009).** Several case-control studies have

also demonstrated a strong association between FLG mutation and early AD onset, disease severity, eczema herpeticum, AD related asthma, and allergen sensitization **(Schuttelaar *et al.*, 2009).**

1.2.2 Adherent junctions (AJ)

Desmosome are known as macula adherens, which is a cell structure specialized for cell-to-cell adhesion. They belong to the cadherin family of extracellular transmembrane glycoproteins and provide structural integrity to the CE. Desmoglein (DSG) and desmocollin (DSC) are desmosomal proteins both linked to the keratin filaments through corneodesmosomal plaque proteins **(Fig 1.3).** DSG and DSC play an important role in maintaining a functional skin barrier, and can be affected by *S. aureus* proteases with a particular susceptibility demonstrated in atopic dermatitis (AD) (**Amagai *et al.*, 2000).** If the connecting adherent junction of the epidermal cells is not functioning correctly, epidermal cells can pull apart and allow abnormal movements of fluid within the skin, resulting in blisters and other tissue damage. Blistering diseases such as pemphigus vulgaris and pemphigus foliaceus are autoimmune diseases in which autoantibodies target the proteins desmoglein 3 and desmoglein 1 respectively.

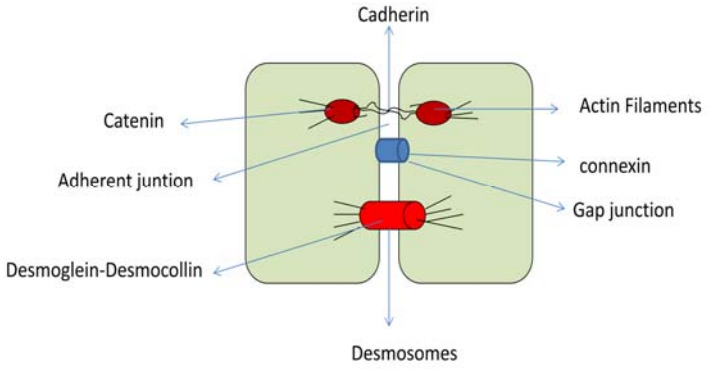


Figure 1.3 Schematic representations of epidermal Adherent junction

Desmosome are the cellular structure of adherent junction consists of two protein Desmoglein (DSG) and desmocollin (DSC). DSG and DSC are linked to the epidermal plaque protein (Actin filaments and Catenin).

1.2.3 Tight junctions (TJ)

The tight junctions (TJ) can play a central role in cell-cell adhesion in simple epithelia and are responsible for connecting neighboring cells in a controlled manner. They establish and maintain tissue barriers for the transport of particles and molecules between different compartments of the cell. Tight junctions maintain normal cell surface polarity by separating the molecular compartment of the apical and basolateral portions of the plasma membrane ("fence function") as well as their role as barrier **(Brandner J *et al.*, 2002).** Claudin family, Occludin and zona occludin (ZO) protein which belongs to the membrane-associated-guanylate kinase homologs (MAGUKs) are the main proteins for the tight junction's structure and functions **(Figure 1.4).** There are two other lesser important proteins including junctional adhesion molecule (JAM) and tricellulin **(Tsukita *et al.*, 2008).**

1.2.4 Claudin proteins

Claudin proteins are the integral component of tight junctions, forms paracellular barrier and controls the flow of molecules in the intercellular space between cells of the epithelium. They have four transmembrane domains with the N-terminal and the C-terminal in the cytoplasm. Claudin-1 was the first member of the claudin family to be identified as a tight junction component **(Swisshelm *et al.*, 2005)**. Claudin proteins have molecular mass (20-27 KDa) and contain four short membrane spanning region a short N terminal cytoplasmic domain, two extracellular loops, one intracellular loop and C terminal cytoplasmic domain **(Tsukita *et al.*, 2002).** Under freeze-fracture electron

microscopy, TJs appears as a network of continuous anastomosing intramembranous strands. These strands consist mainly of claudins and occludin **(Tsukita *et al.,* 2001).** Tissue distribution patterns for the Claudin family members are distinct. Claudin-1 and 3 were expressed in human neonatal and adult keratinocytes as well as in HaCaT keratinocytes, all other tested Claudins were negative **(Brandner J *et al.,* 2010).** Adult skin was positive for claudin-1 in the intercellular space of the spinosum layer and the basal layer but negative for claudin-2 in all skin layers. Claudin-1 and 3 are involved in cell to cell contact between keratinocytes in human epidermis. **(Tebbe B *et al,* 2002).** The Claudin protein family members are predicted to possess four transmembrane domains with intracellular N and C termini. It is suggested that all Claudins share a common transmembrane topology. The MAGUKs family proteins ZO-1 ZO-2 and ZO-3 binds to the COOH-terminal YV sequence of Claudin-1-8 through their PDZ1 domain in vitro **(Itoh M et al., 1999).**

1.2.5 Zona Occludens

The MAGUK-like family of proteins ZO-1 (a 220 kDa protein), ZO-2 and ZO-3 are associated with transmembrane proteins localizing at TJ (Occludin, Claudin) JAM (junctional adhesion molecule) and with adhesion Junction (AJ) through α Catenin **(Muller, S.L *et al.,* 2005).** ZO family proteins are the cross–linker proteins which anchor TJ proteins to the actin cytoskeleton. ZO-1 is structurally different from the other family members by having a ZU5 domain at the C-terminal, although the function of this domain is unknown **(Utepbergenov, D. I et al., 2006)**. An Actin-binding motif appears present in the C terminal half of the molecule and transfection of this end into fibroblasts

caused co-localization of ZO-1 and 2 with actin fibers **(Umeda K et al., 2006).** ZO-1 is a functional component in the cadherin-based cell adhesion system, which point towards ZO intercellular adhesion and communication **(Umeda K et al., 2006).** As TJ exist in the confluent epithelial-endothelial cells that's why ZO-1 protein is also expressed under the condition where the TJ are present.

1.2.6 Occludin

Occludin is a 522 amino acid polypeptide with a molecular weight of 65 kDa an important TJs protein encoded by the OCLN gene. Occludin is the main component of TJs and its localization at the TJs was first time described by Shoichiro Tsukita in 1993 together with Claudin group of proteins **(Furuse M et al., 2002).**

Occludin appears to span the plasma membrane four times, forming two extracellular loops and exposing its NH2 and COOH terminus to the cytosol. Interaction of Occludin with several cytoplasmic proteins has been found to occur via its COOH terminus, while the extracellular loops are thought to be involved in the regulation of paracellular permeability and cell adhesion **(Peng, Bi-Hung et al., 2003).**

Occludins are well known to regulate junctional organization and participate in the restriction of the paracellular transport pathway. Loss of Occludin attenuated activation of phosphoinositide 3- kinase (P13K), leading to disorganization of the actin cytoskeleton and reduced cell protrusion. Data indicate that Occludin is required for the leading- edge localization of polarity protein APKC-Par3 (atypical protein kinase C Par3

complex) and promote cell protrusion by regulating membrane- localized activation of P13K **(Du, D et al., 2009).**

1.2.7 Cytosolic proteins

Caveolae (Cav) are 50-100nm flask-shaped invagination rich in cholesterol and sphingolipids described by Palade and Yamada in 1950's. Caveolae are a subset of lipid rafts found on the plasmalemmal membranes of a variety of cells including endothelial, smooth muscle, epithelial cells, and fibroblast. One of the major functions of caveolae is to serve as a platform and to compartmentalize the signaling molecules that reside in or are recruited to caveolae. Caveolae are also involved in transcytoses, endocytosis, and regulation of cell proliferation, differentiation, and apoptosis via a number of diverse signaling pathways **(Frank PG et al., 2003).**

Cytosolic proteins are existed as a plaque, that crosslink junctional membrane proteins and connect TJs to the actin cytotoskeleton. Within this plaque are signaling molecules and transcription factors that signal between TJ and the nucleus, which is important during cellular differentiation as TJ is intimately related to cell differentiation **(Shin at al., 2006).**

Caveolin-1 (Cav-1) 22 KDa is the major constitutive protein of the caveolae that interact with and regulates several proteins including Src family of kinases, G-proteins (a-subunit), G protein coupled receptors, protein kinase C (PKC), eNOS, integrin and

growth factor receptors such as vascular endothelial growth factor recptors (VEGF-R) and epithelial growth factor receptors (EGF-R). Caveolin-1 protein stabilizes these proteins by acting as a scaffolding protein, links Integrin- subunits to the tyrosine kinase FYN, an initiating step in coupling, integrin's to the Ras-ERK pathway and promoting cell cycle progression **(Frank PG et al., 2003).** Transforming growth factor- beta (TGF-beta) signaling proceed from the cell membrane to the nucleus through the cooperation of the type I and II serine/Threonine kinase receptors and their SMAD effectors **(Razani, B et al., 2001).** The TGF-beta cascade is associated with Cav-1 in caveolae and interacts with the type I TGF-beta receptor. Cav-1 is able to suppress TGF-beta mediated phosphorylation of Smad-2 **(Razani, B et al., 2001).**

1.2.7 Functional importance of tight junction

The structure and function of the TJ proteins are very important and dysfunction of these proteins can result in impaired skin barrier integrity. The gate and a fence are the two important functions of tight junctions. The gate function refers to the capacity of tight junctions to regulate the passage of ions, molecules and water through the paracellular pathway. The gate function can be detected by measuring the transepithelial electrical resistance (TEER) of the tissue. The fence function refers to the ability of TJs to restrict the movement of lipids and proteins within the membrane from the apical to the basolateral domains. This function maintains the polarity of the plasma membrane and thus allows the transport of molecules across the epithelia **(Hartsock A et al., 2008)**. The fence function of the TJ is evaluated in monolayers cultured in transwell filters, by

inserting a fluorescent liquid into the apical membrane and detecting if any fluorescent label reaches the bottom part of the transwell.

Claudin-1(CLDN-1) is the most important protein of the TJ, which involved in epidermal barrier function of the skin **(Furuse *et al.*, 2002).** Furuse et al., developed a CLDN-1 knock down mouse, which died within 24 hours of birth due to severe defect in the epidermal barrier even though histologically stratum corneum was normal **(Furuse *et al.*, 2002).** The CLDN-1 gene polymorphism was reported as a possible candidate mutation in AD by De Benedetto (**De Benedetto A *et al.*, 2011).** They used haplotype tagging to show a genetic link between AD and CLDN-1, it was found that CLDN-1protein expression was reduced in the non-lesional skin of patients with AD**.**

One study clarifies the role of TJs function as a barrier during bacterial cell infection. The expression of TJs protein was investigated during infection with *S.aureus*, where delocalization of epidermal TJs proteins was determined. The relocalization of TJs protein in response to *S.aureus* infection may suggest, TJs probably contribute to both inside-out and outside-in barrier function of the skin (**Ohnemus U *et al.*, 2008).** Using HaCaT and porcine ex vivo infection models, CLDN-1- CLDN-4, Occludin and ZO-1 were shown to be down regulated after *S.aureus* infection. This was studied by measuring TEER indicating the functional importance of CLDN-1. In addition almost complete loss of atypical Protein kinase C was demonstrated which plays a key role in TJ formation (**Ohnemus U *et al.*, 2008).** Formation of tight junction strands, which are crucial for this barrier, involves the polymerization of claudins and TJ adhesion molecules, in temporal and spatial manners. The most important function of ZO is to polymerize Claudin at the

adherent site **(Umeda.K *at al.,* 2006).** Epithelial cells deficient in both ZO-1 and ZO-2 were well polarized but did not form TJs due to the lack of Claudin polymerization **(Umeda.K *et al.,* 2006).** Ohnemus found that *S.aureus* secreted proteases down regulated TJ/AJ and desmosomal proteins, including atypical protein kinase (aPKC) at the cell-cell border. It was observed that reduction of the proteins at the TJ/ AJ cause decrease in the trans- epidermal resistance of keratinocytes monolayer in a time and concentration dependent manner. **(Ohnemus U *et al.,* 2008).** Nina Kirschner and Brandner described that barrier for diverse ions and molecules of different size are formed in keratinocytes by TJs and TJs proteins CLDN 1, CLDN 4, Ocln and ZO-1 contribute to this barrier. TJs also form a barrier for water but CLDN 1 and CLDN 4 are dispensable for this barrier, however, CLDN 1 influences important components of the SC barrier **(Kirschner, N et al. 2013).**

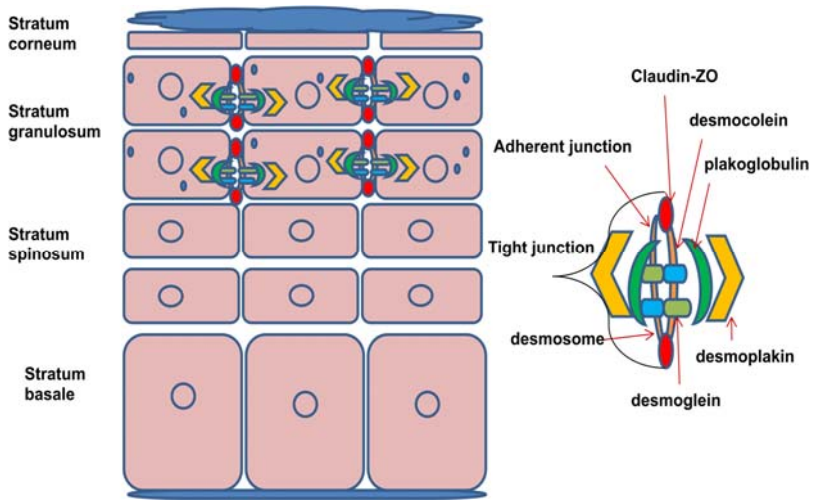


Figure 1.4 structural components of skin tight junction and adherent junction

Tight junction protein Claudin, Occludin and adhesional junction desmosomal proteins desmoglein and desmocollin interact within intercellular space and intracellular cytoskeleton to make a semi-permeable barrier.

1.3 Skin classification scales

The skin can be classified into different types according to the responses it shows to various factors.

1.3.1 The Fitzpatrick scale

This is a numerical classification which classifies the response of different types of skin to UV (Ultraviolet) light. This scale provides basic tool for dermatological research, measures genetic disposition and reaction to sun exposure relating to the color of skin. The Fitzpatrick classification range separates skin type on a scale of 1- 6 as detailed below. The Fitzpatrick skin phenotype classification remains the gold standard for the response of different types of skin to UV light. It is simple and user friendly, however, the system fails to accurately predict skin reactions to insult, injury and inflammation for individuals within a specific skin type class.

Table 1.1 Fitzpatrick skin phenotype classifications (Fitzpatrick TB *et al.*, 1988).

Skin type	Description	characteristics	Score
1	Always burns in sunlight, never tans	Pale, Fair, Freckles	0-6
2	Usually burns, sometime tans	Fair	7-13
3	May burn, usually tans	Light Brown	14-20
4	Rarely burns, always tans	Olive Brown	21-27
5	Moderate constitutional pigmentation	Brown	28-34
6	Marked constitutional pigmentation	Black	35+

1.3.2 The Roberts skin type classification

This classification identifies patient skin type characteristics, and provides a mechanism to predict specific skin type likely response to insult, injury and inflammation **(Table 1.1) (Roberts et al., 2008).** Roberts's skin classification system can uniquely help to predict- Response to the treatment, Clarify post procedure expectation, Optimize outcomes.

It can be a predictor of impending complications such as hyperpigmentation, and scarring. Roberts hyperpigmentation Scale is a 7-points system, measures the natural history of post inflammatory pigmentation in an individual and likelihood to incur a pigmentation problem. This value is based on past medical history, clinical exam, and ancestral background.

Roberts hyperpigmentation (H) Scale: propensity for pigmentation

[H.sub.0]			hypopigmentation
[H.sub.1]	Minimal and transient	(< 1 year)	hyperpigmentation
[H.sub.2]	Minimal and permanent	(> 1 year)	hyperpigmentation
[H.sub.3]	Moderate and transient	(< 1 year)	hyperpigmentation
[H.sub.4]	Moderate and permanent	(> 1 year)	hyperpigmentation
[H.sub.5]	Severe and transient	(< 1 year)	hyperpigmentation
[H.sub.6]	Severe and permanent	(> 1 year)	hyperpigmentation

The Roberts Scarring Scale is a patient's pattern of scarring is classified in this 6-point Scale. Individual scores on the scarring scale help to determine short-term and long-term effects of numerous medical treatments and procedures.

Roberts Scarring (S) Scale describes scar morphology

[S.sub.0] **Atrophy**

[S.sub.1] **None**

[S.sub.2] **Macule**

[S.sub.3] **Plaque within scar boundaries**

[S.sub.4] **Keloid**

[S.sub.5] **Keloid nodule**

To determine a patients profile using the Roberts skin type classification system, clinicians first take each element and assign a numerical value, or "feature" to the element.

1.3 Skin functions

1.3.1 Important functions of the skin

Protection

Skin provides Protection against micro-organism, UV, toxic agents and mechanical insults.

Barrier function

The primary role of the skin is to provide a barrier function. The skin is the first barrier against environmental stresses and pathogens. Protection is provided by the epidermis, which is the outer layer of skin. The epidermis has a broad set of protective barrier functions, which prohibits permeabilization of the environmental allergens **(Groschwitz *et al.*, 2009).** Skin barrier prevents body loss of water and electrolytes and entry of environmental toxin and allergen **(Kalinin AE et al., 2002**). When this barrier is impaired the person is at risk of dehydration and infection. The skin protects the body from environmental injuries and insults. The skin developed a unique immune response and fundamental ability to repair the barrier quickly **(Elias, PM *et al.*, 2007).** Along with the barrier function skin has other important functions which are the following.

Sensation

An important function of the skin is to detect the different sensations of heat, cold, pressure, contact and pain. Sensation is detected through the nerve endings in the dermis which are easily affected by wounds. This sensation in the skin plays a role in helping to protect us from burn wound.

Thermoregulation When the body's heat production is stable, the blood flowing into the dermis is regulated depending on changes in ambient temperature. The purpose of this

regulation is to ensure that the difference in temperature between the skin's surface and the environment remains constant, thus regulating heat loss and maintaining an almost constant body temperature. When heat production increases, blood flow to the dermis also increases, which in turn, increase the heat lost from the skin to the same rate as the excess heat production.

Endocrine function the skin

Skin is one of our main source of Vitamin- D

Synthesis of Vitamin D

By the action of ultra violet light Vitamin-D synthesis on certain parts of the skin **(Webb *et al*., 1989) (Bikle DD et al., 2010).** The traditional pathway of Vitamin-D synthesis and processing involves pre-Vitamin D3 formation in the skin **(Figure 1.5) (Chen *et al*., 2000).** Where it binds with the DBP (Vitamin D binding protein) and is transported to the liver, where it is enzymatically hydroxylated to generate 25OH D3. DBP bound 25OH D3, is then transported to the kidney and is finally hydroxylated by CYP27B1 to hormonally active Calcitriol 1, 25 dihydroxyvitamin D3 (1, 25 (OH) 2 D3)) transported in the circulation after binding with DBP **(Lehman *et al.,* 2001).** However, more recently both hydroxylation steps were found to be able to occur within other cells, including the keratinocytes. The keratinocyte represents the only cell type where the complete enzymatic machinery for the synthesis of 1, 25 (OH) 2D3 from 7-dehydrocholestrol (7DHC) is present and where the synthesis of 1, 25 OH D3 from 7DHC has been shown **(Seifert M *et al*., 2009).**

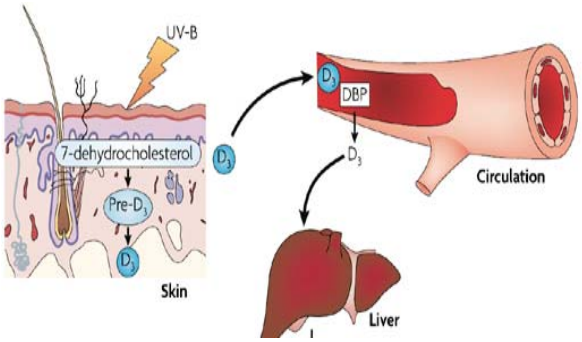


Figure 1.5 Photochemical synthesis of vitamin D3 in the skin

Vitamin D synthesis occurs cutaneously where pro-vitamin D3 (7- dehydrocholesterol) received from the circulation is converted to pre-vitamin D3 in response to UVB exposure. Vitamin D3 obtained from the isomerization of pre-vitamin D3 in the epidermal basal layers or intestinal absorption of natural and fortified foods and supplements, binds to vitamin D-binding protein (DBP) in the blood stream, and is transported to the liver **(Deeb, K. K et al., 2007)**

Antimicrobial peptide expression (AMP)

Normal human keratinocytes revealed a dose-dependent increase of human beta defensins 2 & 3 hBD2, hBD3 and cathelicidin mRNA after UV radiation **(Glaser *et al*., 2009).** UV-B radiation induces the expression of antimicrobial peptide (AMP) in human keratinocytes in vitro and in vivo **(Harder *et al*., 2009).** Thus UV-B induced Vitamin-D can cause increase expression of hBD2 and cathelicidin by stimulating VDR **(Figure 1.6)** (vitamin D receptors). Oral vitamin D supplementation induces cathelicidin production in normal skin **(Tissa et al, 2008).**

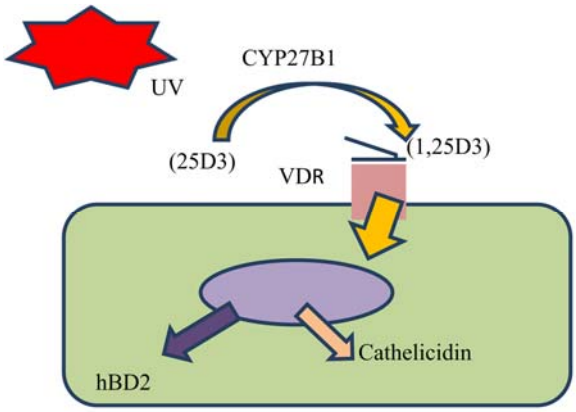


Fig 1.6 UVB triggered vitamin D3 expressed hBD2 and Cathelicidin

In keratinocyte, hydroxylation steps by CYP27A1 and CYP27B1 result in active 25 Vitamin-D3 into 1, 25 OH D3 which activate VDR (Vitamin D receptors). VDR binds to VDRE in the antimicrobial peptide promotor region and cause activation of Cathelicidin and hBD2.

1.4 Atopic dermatitis (AD)

Atopic Dermatitis (AD) is a chronic inflammatory disease, with both genetic and environmental factors implicated in its pathogenesis. Individuals with AD have a defective skin barrier which makes them more susceptible to *S. aureus* colonization and to having pathogenic responses to allergens from dust mites **(Breuer, K et al., 2002)**. In addition, *S. aureus* secreted proteases can further aggravate barrier dysfunction, rendering individuals with AD more susceptible to the epidermal penetration of allergens **(Cork *et al.*, 2009).** Atopic dermatitis most often begins in childhood before age 5 and may persist into adulthood. It is estimated that 75 % of the cases of AD improve by the time children reach adolescence, whereas 25 % continue to have difficulties with the condition through adulthood. AD is known as infantile eczema when it occurs in infants. Infantile eczema may continue into childhood and adolescence presenting with an oozing, crusting rash. This occurs mainly on the scalp and face but can occur anywhere on the body. Individuals with AD are considered "sensitive"; reacting abnormally and easily to irritants, food, and environmental allergens. The most common food hypersensitivity being to eggs, peanuts and milk, ingestion of these can cause a flare in eczema which occurs in about 10% of AD patients less than 2 years old. **(Kim *et al.*, 2013).**

1.4.1 Clinical manifestation

Symptoms of atopic dermatitis may vary from person to person, but most individuals with AD usually present with atopy and pruritus **(Correale CE *et al.*, 1999).**

1.4.1. A Atopy

Atopy is a syndrome characterized by a tendency to be hyper-allergic. A person with atopy presents with a triad of the following, AD eczema, allergic rhinitis (hay fever) allergic conjunctivitis and allergic asthma. The individual component of atopy is caused by a type I hypersensitivity reaction. Atopic responses appear after the body is exposed to various allergens, pollen, dander, food, dust mites, chemicals or irritants **(Burk W et al., 1998).** The presence of an elevated level of total and allergen specific IgE in the serum leading to positive skin prick test to common allergies. Th1 cells secrete IL-2 and IFN-Ɣ and promote cellular immunity and the production of IgG1. Th2 cells secrete IL-4, IL-5, IL-10 and IL-13 and thereby induce the production of IgE and promote eosinophil-mediated inflammation **(Burk W et al., 2008)**. These results suggest that atopy refers to the potential for producing inflammatory reactions in various organ systems, primarily initiated by Th2 cytokines and that Th1/Th2 and non-Th proinflammatory cytokines are all involved in the disease process due to Th1/Th2-cell reversal **(Chen, L et al., 2004).** The Th2 predominance in atopic individuals results in production of IgE antibodies and attracts eosinophils with specific high affinity IgE receptors. This Th2 cell activity leads to release of IL-4, IL-5, IL-13 and IL-3. **(Akdis *et al.*, 1999; Dworzak *et al.*, 1999).**

1.4.1. B Pruritus

Pruritus (or itching) in individuals with AD is considered to be the most disturbing symptom. Once their itch begins, it increases sensitivity of the surrounding skin which reacts to light stimuli with more severe itch. The most commonly recognized triggers are

dry skin, heat and perspiration, irritants (wool or synthetic fibers, soap and detergents, some perfume and cosmetics, cigarette smoke), allergens such as (pollen, pet dander, dust, certain vasodilator foods), emotional stress, alcohol, upper respiratory tract infections and house dust mite allergens **(Bieber T et al., 2008).** Proteases, kinins, prostaglandins, neuropeptide, and acetylcholine can all cause itch or potentiate the release of histamine **(Ui H *et al.*, 2006)** and may be significant in this condition.

Various central and peripheral mediators have been suggested to play a role in the pathophysiology of itch of AD patients. The sensitization of Peripheral and central nerve fibers contribute significantly to triggers itch in AD. Several mediators have been described as associated with itch in AD, including serine proteases, interleukin 31 and nerve growth factor **(Yosipovitch G et al., 2008).** Itch fibers are sensitized peripherally due to increased concentration of nerve growth factor (NGF) in the inflamed area that could lead to sensitization of the central itch process. Sensation provoked by scratching can be perceived as an itch by the patient and thus excerebrate pruritus **(Groene *et al.*, 2001).**

1.5 Pathophysiology of atopic dermatitis

1.5.1 Epidermal Barrier Dysfunction Epidermal barrier dysfunction is fundamental to the pathogenesis of AD, but remains incompletely understood **(Cork *et al.*, 2009).** The epidermal barriers comprised of three components, stratum corneum (SC), tight junctions (TJS), and Langerham cell system (LCS) as an immune barrier. As SC is in direct contact with the external environment, the SC defends against pathogen invasion

by creating both a physical and chemical shields mainly comprising lipid and antimicrobial peptides (AMPs) respectively **(Aberg *et al.*, 2008).** The strong association between both genetic barrier defects and environmental insults to the barrier with atopic dermatitis suggests that epidermal barrier dysfunction is a primary event in the development of this disease.

1.5.2 Environmental allergens

AD is an atopic disorder often found to be associated with hay fever and asthma, and associated with generally high IgE levels. Irritants readily have an excitatory effect on the impaired barrier of the skin of an individual with AD. Daily washing with soap and noxious agents can elicit an irritant contact reaction in AD **(Hogan *et al.*, 1994)**, but other specific agents have also been implicated, as described below.

1.5.3 Foods

Food hypersensitivity affects about 6-8% of children under 4 years of age and 2% of the general population beyond the first decade of life **(Sampson *et al.*, 1999)**. AD and asthmatic patients can have anaphylaxis resulting from exposure to specific foods. For these reasons, the role of food as a trigger in all infants with moderately to severe AD should be considered **(Sicherer *et al.*, 2011).** Histamine containing foods such as cheeses, vegetables (tomatoes, eggplant, and spinach), fish, shellfish, red wine and grapes can cause skin dryness and flushing leading to pruritus **(Spencer *et al.*, 1994).**

1.5.4 House dust mites House dust mite species, Dermatophagoides pteronyssinus (Der p-1) and Dermatophagoides farina (Derf-1) are the source of the major house dust

mite (HDM) allergens, detected on the surface of human skin, and have been proposed to play a role in the development of AD **(Friedman *et al.,* 1999).** Der p1 and Der f 1 are cysteine proteases that elicit IgE responses in 80% of mite-allergic subjects and have proinflammatory properties. Der p1 protease weakened not only the barrier function of the skin but impaired the epidermal barrier recovery in both human and murine skin **(Nakamura *et al.,* 2006).** Repeated topical application of HDM extracts in a sensitive mouse model for AD triggered strong skin inflammatory responses, through inflammatory cell infiltrate in the upper dermis and elevated IgE concentration **(Oshio *et al.,* 2009**).

1.5.5 Climate- effect

Individuals with AD have an abnormal pattern of thermoregulation, which is believed to reflect an intrinsic disturbance of the parasympathetic system, which influence the pathogenesis of AD**.** Epidemiological studies suggest that climate influences the prevalence of atopic eczema. Atopic eczema has been reported worldwide to be positively associated with latitude and negatively with temperature (**Weiland SK et al., 2004)**. Consequently, seasonal variation can impact the disease and most individuals with AD are improved in summer but worsen in winter. However climatotherapy can play significance role in the treatment of atopic dermatitis, which is cost-effective **(Harari M et al., 2000).**

1.5.6 Infectious agents -The altered skin barrier of atopic patients provides a portal of entry for various pathogens (**Leung** *et al.,* 2001).

1.5.6 A Bacteria

S. aureus colonization can be demonstrated in over 90% of lesions in individuals with AD. The density of S. aureus on inflamed AD lesions without superinfection can reach up to 10^7 colony forming unit (CFU) per cm² on lesional skin. One study suggests that a strategy by which S. aureus exacerbates or maintains skin inflammation in AD is by secreting a group of toxins known to act as superantigens, which stimulate marked activation of T-cells and macrophages. (Leung et al., 2001). *S.aureus* secretes, cysteine (metallo) and serine proteases, these extracellular proteases are also proposed to have role in the virulence of this disease.

1.5.6-B Viruses

Individuals with AD appear to have reduced immunity to herpes virus; eczema herpeticum (EH) is more commonly seen in infants and children with AD. Most cases of EH are due to Herpes simplex virus type 1 or 2, other viruses may be responsible, such as coxsackie virus however varicellar form eruption 'eczema herpaticum' caused by spread of herpes is recognized as a potentially dangerous complication of AD **(Wollenberg, A et al., 2003)**. Eczema herpaticum also occurs when there are other reasons for the breakdown of skin barrier including, burn, pemphigus vulgaris, Darier disease, Cutaneous T cell lymphoma, Ichthyosis vulgaris.

1.6 Diagnostic criteria

Atopic dermatitis has a wide spectrum of dermatological manifestations and there is disagreement about its definition. Nevertheless, results and reproducibility of genetic, etiological, epidemiological, diagnostic and therapeutic studies depend on establishing reliable and valid diagnostic criteria. During the past decades various lists of diagnostic criteria for AD have been proposed **(Table 1.2) (Brenninkmeijer, E. E et al., 2008).**

1.6.1 Hanifin and Rajka diagnostic criteria for Atopic Dermatitis

Hanifin and Rajka developed guidelines for the diagnosis of atopic dermatitis in 1980, to define a disease for which an objective diagnostic laboratory test was lacking and for which nomenclature was inconsistent. According to Hanifin and Rajka, the diagnosis of atopic dermatitis requires the presence of at least three of four major criteria and three of 23 minor criteria.

Major criteria: must have three of four

1-Puritis 2- Dermatitis affecting flexural surfaces in adults. Facial and extensors surfaces involvement in infants and children 3- Chronic relapsing dermatitis 4-Personal and family history of Atopy (asthma, allergic rhinitis, atopic dermatitis).

Minor criteria: should have three out of 23-1-Xerosis,2-Ichthyosis, palmar hyperlinearity or keratosis pilaris,3-Immediate type -1 skin test reactivity,4-Raised serum IgE,5-Early age of onset,6-Tendency toward cutaneous infection,7-Nipple eczema,8-Cheilitis,9-Recurrent conjunctivitis,10-Dennie-Morgan infraorbital fold,11-Kerato

conus,12 -Facial erythema,13-Pityriasis alba,14-Itch when sweat,15-Food intolerance,16-Delayed blanch or white dermographism,17-Course influenced by environmental or emotional factors 18 Intolerance to wool and lipid solvents 19 Anterior neck fold 20 intolerance to wool and lipid solvents 21 perifollicular accentuation 22 Orbital darkening 23 Anterior subcapsular cataract.

D. Exclusions: Firm diagnosis of AD depends on excluding conditions such as scabies, allergic contact dermatitis, seborrheic dermatitis, cutaneous lymphoma, ichthyoses, psoriasis, and other primary disease entities.

1.6.2 The UK diagnostic criteria of atopic dermatitis

A UK working party set about the task of developing a minimum list of reliable discriminators for AD, using the Hanifin and Rajka list of clinical features as the building blocks. In addition to validity, repeatability and simplicity, a further requirement of the definition was that it should correspond well to the clinical concept of disease, be applicable to different ages and ethnic groups and be acceptable to subjects under study **(Williams *et al.*, 1995)**. According to UK diagnostic criteria, in order to be diagnosed a case of AD, the child must have an itchy skin condition for the last 12 months. Including itch, the child may have three or more of the following:

1- Onset below age two
2- History of flexural involvement
3- Personal history of asthma or hay fever
4- History of generally dry skin
5- Visible flexural dermatitis

In children aged less than 4 years a history of atopic disease in a first degree relative may be included. The UK criteria have performed well in hospital and community validation studies **(Williams *et al.*, 1996).** In an independent validation study of children attending hospital dermatology outpatients, the criteria were shown to have a sensitivity and specificity of 85 % and 96 % respectively, when compared with a dermatologist's diagnosis **(Williams HC *et al.*, 1999).**

Positive and negative predictive values in this survey were 80 % and 97 % respectively. The criteria appear to be equally applicable to children of different ethnic and socioeconomic groups. They have worked well in children down to the age of 3 years, but further evaluation in younger children and adults is awaited. The criteria is easy to ascertain, and they have proven to be highly acceptable to children and adults because of their relatively simple and non-invasive nature.

1.6.3 Epidemiological uses for the diagnostic criteria:

The commonest epidemiological uses for the diagnostic criteria will be to compare prevalence rates between countries or in the same population at different points in time. As Williams stated that, "Even though the sensitivity and specificity of the UK criteria for atopic eczema appear quite high, the underlying prevalence of atopic eczema has a critical influence on the positive predictive value of the criteria" **(Williams *et al.*, 1995).** Thus, for one disease, there may be range of definition with slightly different validity indices, each of which may be better suited to specific study design or requirements. They all define the same disease, but with different precision and practical suitability for different study design and constraints **(Williams *et al.*, 1995).**

Table 1.2 Diagnostic criteria's of atopic dermatitis

Diagnostic criteria	Features	Hospital based study		Population based study		Positive predictive value	Negative predictive value
		Sensitivity	specificity	Sensitivity	specificity		
Hanifin and Rajka	3majors +3minors	Willims et al. (1999) 88-96%	77.6-93.8%	74%	98%	83.1%	93.5%
Kang and Tian	1major+ 3minors	Gu et al. (2001) 95.5%	100% 89%	77.6%	90.1%	100%	96.0%
Schultz-Larsen	≥ 50 points	Schultz Larsen et al. (1996) (88-94.4%) Laughter et al. (2000)	(77.6-95.9%)	88-94%	77-89%	60.7%	97.4%
UK diagnostic	Pruritis +3 minors	Williams et al. (1999) (10-100)	(89.3-99.1%)	69.5%	92%	80 %	97 %

1.7 Prevention

Individuals with AD need to keep their skin hydrated through proper bathing and use of moisturizers to avoid it to becoming dry. This can best be achieved by applying a moisturizer right away after bathing, even while the skin is wet. However irritants such as soap, perfumes, noxious agents and wool, which are common triggers, should be avoided **(Hogan et al., 1994**), in addition to avoiding certain food (e.g. eggs, peanuts, and milk and soy products) that can cause a rash or make a rash worse.

1.7.1Tolerance against foreign antigens

The immunological phases of food allergy in AD include a sensitization phase and an effector phase. Response to that allergen or tolerance occurs in the effector phase **(Noh *et al.,* 2011)**. Tolerance can be classified into central and peripheral **(Schwartz *et al.,* 1993).** Central tolerance is a process of negative selection of the developing T cells in the thymus, whereby T cells with high affinity for self-antigen are eliminated in the early stages of differentiation **(J Sprent *et al.*, 2002)**. Whereas, peripheral tolerance requires then development of regulatory T-cells, which play essential roles in the maintenance of peripheral tolerance by continuous suppression of auto reactive T-cells that escape central tolerance mechanism **(Seddon *et al.*, 1999; Suri *et al.,* 1998).** Peripheral tolerance has been divided into high dose and low dose responses **(Rocha *et al.,* 1991)**. High dose responses occur via anergy of T-cells and T-cell deletion by apoptosis **(William *et al.*, 2005),** whereas low- dose tolerance to antigens administered repeatedly, is achieved by active suppression of T-effector cells by regulatory T-cells **(Prakken *et al.,* 2004).**

The route of exposure to antigen may play an important role in the development of tolerance. Tolerance induced by high-dose allergen-exposure via skin was associated with rapid switch and expansion of IL-10 producing regulatory T-cells **(Meiler. F *et al.*, 2008).** Oral immune therapy appears to be effective in inducing desensitization in most patients, as well as oral tolerance in a subset of patients with food allergy **(Beyer K et al.,2008) (Weiner HL *et al.*, 1997; Chehade M *et al.*, 2005).** T-cells and IL-10/TGF-beta appear to be the major players in natural and acquired tolerance to food antigen **(Aneta *et al.*, 2011).**

1.7.2 Avoiding contact with house dust mite (HDM)

HDM is a very common allergen and contact with HDM can cause exacerbation of the disease; especially on face, head, neck and hands, areas which are direct in contact with mites **(Darsow *et al.*, 1996).** HDM proteases Der P 1 and Der f 1 not only weakened the skin barrier but also impaired the epidermal barrier function repair. HDM allergen plays a role in the development of AD. Repeated topical application of HDM extract in mice, a sensitive animal model for AD exacerbates the disease. In 1932 Rost was the first to report that patients with AD improved when they were placed in a dust-free environment **(Kumei *et al.*, 1995).** Consequently, this contact should be avoided. The hands are frequently involved in individuals with AD and present unique physical, social and therapeutic challenges for patients. The involvement of dorsal hand surface and the volar wrist may suggest atopic dermatitis as a contributing etiological factor. **(Simpson *et al.*, 2006)**

1.7.3 Avoiding stress; Stress has been recognized as an important factor in the pathogenesis of AD. The responses to stress are variable and dependent on the existing psychological condition of the patient and the family. The stressful events often have been observed to occur before an AD exacerbation **(Bender, B. G et al., 2002).**

1.8 Treatment -1.8.1 Skin barrier protection

The primary treatment for AD involves skin barrier protection, in conjunction with preventative measures, such as avoiding or minimizing contact with known allergens. To combat the severe dryness associated with AD, moisturizer should be used daily. Moisturizers should not have any ingredients that may further aggravate the condition. Creams are thicker moisturizer than Lotions, which has low viscosity and are thinner moisturizer. Creams retained moisture better and typically work well on dry skin, prevented transepidermal water loss **(Elmariah SB et al., 2011).** AD is also linked to a deficiency in ceramide, which is the lipid that comprises the skin barrier. The stratum corneum ceramide deficiency is possibly the cause of abnormal barrier function. One of the most promising barrier repair methods has been ceramide-dominant physiological lipid based barrier repair topical emulsions **(Chamlin *et al.*, 2002).** In contrast to the traditional moisturizers, these formulations focus on the physiological lipid replacement therapy, particularly ceramides, to restore normal balance of the epidermal barrier **(Kang *et al*., 2008).** Ceramide-based moisturizers have been shown to be beneficial in reducing TEWL, improving barrier function, and thus, can be a useful component in AD management **(Ishida *et al.*, 2012).** The features of occlusive, humectant and emollient moisturizers are summarized in **(Table 1.3)**.

Table 1.3 Different types of moisturizers

Occlusive	Humectants	Emollient
-Reduces TEWL by creating a hydrophobic barrier over the skin and contributing to the matrix between corneocytes	-Able to attract water from two sources: (a) they enhance water absorption from dermis into the epidermis (b) in humid conditions they also help the SC to absorb water from the external environment	-Emollients provide some occlusivity and improve the appearance of the skin by smoothing flaky skin cells
-Most occlusive agents have pronounced effects when applied to the slightly dampened skin	-Many humectants may have emollient properties	-Many different types of emollient ester and oils are available.
-Their main limitations include odor and potential allergenicity	-Enhance desquamation by digesting desmosomes	-Emollients are generally grouped by their ability to spread on the skin
-Most occlusive agents are associated with a greasy feel	-Glycerol reduces the scaling associated with xerosis	-Emollient lipids similar to those naturally found in the skin may also increase the rate of barrier repair.

1.8.2 Corticosteroids.

Topical steroids are also very important drugs used to control inflammatory skin diseases, particularly dermatitis **(Table 1.4)**. Corticosteroids suppress the multiple inflammatory genes that are activated in chronic inflammatory diseases, by reversing histone acetylation of activated inflammatory genes through binding of glucocorticoid receptors.

In general, individuals should use a potent preparation for a short time (few days and weeks) and a weaker preparation for maintenance between flare ups. Pulse therapy refers to the application of corticosteroids for 2-3 consecutive days each week or two. This is useful for maintaining control of chronic diseases (**Thomas KS et al., 2002).** Generally a milder topical steroid or non-steroid treatment is used on the in-between days. A single whole body application requires 10 g for a baby and 30 g for an adult.

Table 1.4 Characteristics of topical corticosteroids

Class	Potency	Corticosteroids	Side effects
Class 1	**Very potent** (up to 600 times as potent as hydrocortisone)	Clobetasol propionate Betamethasone dipropionate in propylene glycol	More than 50 g of clobetasole propionate or 500 g of hydrocortisone each week can result in adrenal gland suppression in an adult and can cause Cushing's syndrome. (**Coureau B et al., 2008)**.
Class 2	**Potent** (150-100 times as potent as hydrocortisone)	Betamethasone valerate Betamethasone dipropionate Diflucortolone valerate Fluticasone valerate Hydrocortisone 17-butyrate Mometasone furoate Methylepridnisolone aceponate	Skin thinning (atrophy) and stretch marks (striae). Easy bruising and tearing of the skin (fragility). Telangiectasia Peri-oral dermatitis (erythematous papules around the mouth). Contact allergy to the steroids molecule itself or another ingredient-The risk of these side effects depends on the strength of the steroid, the length of application, the site treated, and the nature of the skin problem
Class 3	**Moderate**(2-25 times as potent as hydrocortisone)	Clobetasone butyrate Fluocinolone acetonide Trimcinolone acetonide	
Class4	**Mild**	Hydrocortisone 0.5-2.5% (acetate or alcohol)	

1.8.3 Immunosuppressants

Tacrolimus and pimecrolimus are immunosuppressants which can also be used as a topical preparation in the treatment of severe AD. Immunosuppressants have unpleasant side effects in some patients. Oral immunosuppressants can be prescribed for severe cases that do not respond to other medication.

1.8.3 A-Topical immunosuppressants

The topical immunosuppressants or calcineurin inhibitors pimecrolimus cream and tacrolimus ointment are particularly useful for mild to moderate AD. They are anti-inflammatory but do not thin the skin or affect its barrier function. Topical immunosuppressants are particularly useful for eczema in thin-skinned areas (face, genitals and body folds). Topical pimecrolimus is most effective when used at the earliest sign of eczema redness or itching to prevent flares. Topical tacrolimus is more potent and may have greater immunosuppressive activity **(El-Batawy MM et al., 2009).** The main side effect is initial burning, feeling warm or itching. Other side effect includes skin erythema, flu like symptoms headache and skin infection**.** Results from several pharmacokinetic studies of both pediatric and adult patients with AD demonstrate that tacrolimus was minimally absorbed into the systemic circulation. Tacrolimus does not seem to accumulate, either in the skin or blood following repeated applications **(Reitamo S et al, 2000).**

1.8.3 B- Oral non-steroidal immunosuppressants

If an ongoing oral medication is required a non-steroidal immunosuppressant such as azathioprine; methotrexate, cyclosporine or mycophenolate may be used to enable the dose of steroids to be reduced and eventually discontinued **(Meggitt SJ et al, 2001).** However, monitoring is needed because side effects may occur.

1.8.3 C- Side effects related to immunosuppressant

People having certain medical conditions including shingles, high blood pressure, kidney problems, liver disease, pregnancy and susceptibility to infection should not use immunosuppressants.Azathioprine can cause light sensitivity and severe reactions upon exposure to sunlight and long term uses may increase the risk of skin cancer **(Murphy L A *et al.*, 2002).**

1.8.4 Ultra Violet (UV) exposure

Phototherapy is a one of the therapeutic options in atopic dermatitis. AD can be treated with UVB or PUVA (psoralen+UVA) UVA exposure which has been found to have local immune-modulatory effects on affected tissue, and may be used to decrease the severity and frequency of exacerbations. Phototherapy is reserved for severe eczema as it is expensive, time-consuming and has potential side effects. It involves controlled exposure to UVB either of narrow band or broad band (NB-BB) or UVA for a few minutes two to three times each week. A treatment course may continue for several months. There are some AD patients who do

not get good respond to UVB, or even get worse when get phototherapy. The risk and benefits of UVB (NB & BB) and UVA are summarized in **(Table 1.5).** In conclusion,

narrow band UVB could be considered as the first line phototherapeutic option for moderately severe atopic dermatitis (**Gambichler *et al.*, 2008**).

	Narrow band UVB (NB-UVB)	Broad band UVB	PUVA

		(BB-UVB)	
Definition	Is more common form of phototherapy with specific wavelength of UV radiation 311-312 nm	Traditionally broad band UVB has been used, it has wavelength of UVB radiation 290-320 nm	Photo chemotherapy which is the use of psoralin then expose to UVA.
Uses	Atopic eczema, Psoriasis	Psoriasis, Eczema	Vitiligo-Psoriasis-PLE
Advantages and Benefits	-Exposure times are shorter but higher intensity. Longer remission -Influence on the expression of antimicrobial peptides	-BB-UVB has a significant safety advantage over most other psoriasis therapies-Skin cancer-has not been clearly demonstrated.	- PUVA is an effective treatment for most psoriasis patients, Longer remission -Its effectiveness even in severe psoriasis, allowing patients to restore a high quality of life
Side effects	-The most common side effect of NB-UVB therapy is erythema Long term exposure can cause skin ageing and cancers	-BB-UVB can cause burn -Long term exposure can cause skin ageing and cancers **(SH Silva *et al.*, 2006)**	-PUVA can cause erythema and nausea Patients who undergo PUVA therapy are at a higher risk of skin cancer, and risk increases with the total dose of PUVA received

Table 1.5 Phototherapeutic option for AD and their risks/ benefits

1.9 *Staphylococcus Aureus:*

S. aureus is a facultative anaerobic gram and catalase positive coccobacillus also known as "golden staph". *S.aureus* appears as grape like clusters when viewed through the microscope and has large, round golden –yellow colonies. S. aureus cell wall structures are presented in **(Figure 1.7)** *S. aureus* can cause a range of illnesses from minor skin infections such as boils, impetigo, folliculitis furuncle, carbuncle, scalded skin syndrome and abscesses, to life threating diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia and sepsis **(Hanifin *et al*., 1991) (McCaig LF et al., 2006).**

Each year, some 500 000 patients in American hospitals contract an *S.aureus* infection **(Bowersox *et al*., 2007).** The major concern in the treatment of *S.aureus* infection is the ability of these bacteria to acquire resistance to antibiotics. *S.aureus* virulence is multifactorial, because a broad range of conditions that related to virulence factors. Those factors can allow *S.aureus* to adhere to the surface, invade cell membrane and avoid the immune system and can cause harmful toxic effects to the host. It is estimated that 20 % of the human population are long term carriers of *S. aureus* **(Kluytmans *et al.,* 1997).** Colonization primarily occurs in the anterior nares, Axila, and perirectal area **(Von Eiff *et al.,* 2001).**

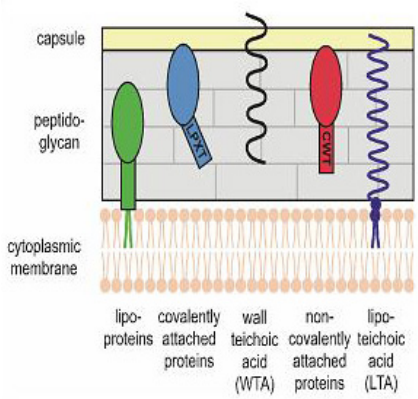


Figure *1.7 S.aureus* cell wall structures

An *S.aureus* cell wall consist of three layers: an outer polysaccharide capsule; peptidoglycan (murem) layer; and inner cytoplasmic membrane. Into this structure, proteins and teichoic acid are embedded and protrude from the cell wall on its outer side, forming a "fuzzy coat". Capsule is thin and may be seen only under the electron microscope. Sometime more bacteria share one capsule and form a slime layer or biofilm, mostly found on the inner wall of venous and urinary catheters. (phil.cdc.gov)

S. aureus nasal carriage is a well-defined risk factor for subsequent infection in nearly all categories of hospitalized patients that has been studied. However studies have been performed to evaluate the effect of eradication of carriage using mupirocin nasal ointment, which have been inconclusive so far in most subgroups **(Kluytmans *et al.*, 2005).**

S.aureus was isolated in 69.7% of the eczematous lesion and in 42.4% of the non-eczematous skin of patient with atopic dermatitis. The nasal carriage rate of *S.aureus* was higher in atopic dermatitis patients (51.5%) than in non-atopic (35%) **(Goh CL et al., 1997).** *S.aureus* secrete proteins that inhibit complement activation and neutrophil chemotaxis or that lyse neutrophils, neutralizes antimicrobial peptides.

Furthermore, *S.aureus* expresses several types of superantigen that corrupt the normal humoral immune response, resulting in anergy and immunosuppression **(Foster, T. J et al., 2005).** *S.aureus virulence* factors are discussed in **(Table 1.6).** *S.aureus* exacerbates AD by acting as a superantigen, stimulating an augmented Tcell response and promoting increase production of IgE. The elevated IgE levels contribute to immune dysregulation (**Leung, D et al., 2001)**

Table 1.6 *S.aureus* virulence factors

	Virulence factors	Function
Extracellular adherence protein (EAP)	- Protein A (SPA), - Fibronectin binding protein A and B - Collagen binding protein, - Clumping factor (Clf) A and	(EAP) is an adhesion which enables *S.aureus* to adhere to host cells of another organism, and an invasion facilitating its internalization into these cells.
Toxins	Superantigen . 1-Toxic shock syndrome (TSS-1). 2-Enterotoxins (SE-(A, B, C, D, E, G, H, I). Cytolytic toxins-A-hemolysins α,β,Ɣ, B- leukocidin- panton-valentine leukocidin -Disease causing toxins Exfoliative toxins (ET) - is associated with scalded skin syndrome (SSS)	Target specificity varies. Induce apoptosis (at low concentration) and lysis of various cell types, including erythrocytes, lymphocytes, monocytes, epithelial
Enzymes	Lipases, hyaluronidases , staphylokinase,exfoliative toxin, serine, cysteine,aureolysin	Degradation of hyaluronic acid, Plasminogen activation; inactivate ,antimicrobial peptides-Act as serine ,proteases; activate T cells, Inactivate neutrophil proteolytic activity; ,inactivate antimicrobial peptides
Chemotaxis Inhibitors protein of S.aureus	(CHIPS) is an exoprotein produced by several strains of *S.aureus*	(CHIPS) Inhibit complement and a potent inhibitor of neutrophil and monocyte chemotaxis towards C5a and formylated peptides like fMIL.

1.9.1 *S. aureus* secreted proteolytic enzymes

S.aureus is a gram positive and catalase positive cocco bacillus secrete extracellular proteases which are produce in the form of pre-proenzyme require activation in the extracellular meilloi. *S.aureus* secreted lipases, hyaluronidase, collagenase and nucleases are proteases, which convert host tissue into nutrient for bacterial growth.

The major proteolytic enzymes secreted by *S. aureus* are metalloprotease (aureolysin), serine glutamyl endopeptidase (SspA; also known as V8, six Spl (serine like protease) and two related cysteine protease referred to as staphopain A (Scpa) and cysteine protease SspB **(Table 1.7) (Arvidson *et al.,* 2001).** The characteristic and function of extracellular proteases are described in the **(Table 1.7)**. These extracellular proteases are secreted in the inactive zymogen form and after activation proposed to have roles in the virulence of these bacteria. The activation of *S. aureus* extracellular proteases are presented schematically in **(Figure 1.8)** where *S.aureus* proteases, Aur, SspA, Ssp B and ScpA are secreted as a zymogen form. *S.aureus* six Spl proteases are secreted as an active proteases, analysis of the Aur reveals that it is cleaved between Glu and an Ala residue, which is a characteristic of endopeptidase SspA and Spl proteases. As mutation of SspA does not affect the activity of Aur, it is possible that Aur achieves catalytic maturation in a Spl-protease-dependent manner, this is because Spl proteases have no requirement for proteolytic maturation **(Reed.S et al., 2001). Se**veral in vitro studies suggested these proteases are the virulent factor **(Shaw *et al.,* 2004 Potempa J et al.,2009)**. In addition, *S. aureus* secreted proteases can aggravate the antimicrobial barrier by degrading adhesion junctions (Amagai ***et al.,* 2000).**

Activation of *S.aureus* secreted proteases

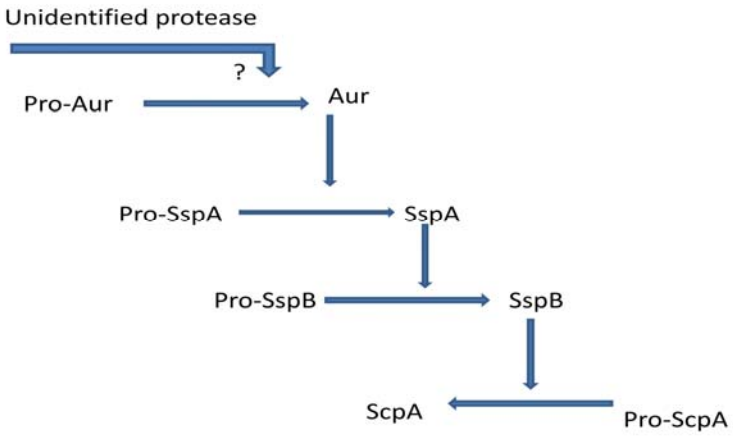


Figure-1.8 Activation of *S.aureus* extracellular proteases.

The cascade of activation of extracellular proteases is proceeds from aureolysin (Aur), serine glutamyl endopeptidase (SspA), Cysteine proteases A & B (SspB and ScpA). *S.aureus* proteases are secreted as inactive zymogen (Pro) that must undergo processing for full activity. The Pro-Aur auto-activates allowing for processing of SspA then it proceeds from V8 (SspA) to staphopain B (Ssp B) and ScpA**. (Lindsey Shaw et al., 1999) (Popowicz GM et al., 2006)**

Regulation of *S. aureus* virulence factors

The accessory gene regulator (Agr) of *S. aureus* is the central regulatory system that controls the gene expression for a large set of virulence factors. The quorum sensing system is the major controlling factor which controls the expression and activity of these virulence factors. The quorum sensing system is also referred as accessory gene regulator (Agr) which are presented in the **(Figure 1.9)**. The Agr locus consists of two transcripts: RNA II and RNA III encode four genes (Agr, A.B.C.D) whose gene products assemble a quorum sensing system **(Saenz, H. L. et al., 2000).** This complex regulatory mechanism ultimately functions via the RNAIII product, generated from one of the two divergent promoters of the Agr operon, p3. The other promoter, p2, encodes the Agr structural genes, Agr BCDA. AgrB is a transmembrane transport protein with endopeptidase activity and facilitate AgrD cleavage, followed by secretion of the resulting octapeptide subsequently termed the auto-inducing peptide (AIP). AgrC forms a membrane-bound histidine kinase, which senses external levels of AIP. These binding events results in auto phosphorylation of AgrC, followed by transfer of this phosphate to the intracellular AgrA protein. AgrA is a DNA-binding protein which leads to activation of the p2 and p3 promoters, as well as direct activation of specific toxin genes (**Cheung AL et al., 2002)**. This process is required to diversify the composition of cell surface proteins and to transform the bacterial phenotype from one of adhesion, expressing a variety of cell surface proteins responsible for binding to host tissues, into one of invasion, producing factors which degrade tissues and allow for the spread of infection from initial colonization foci.

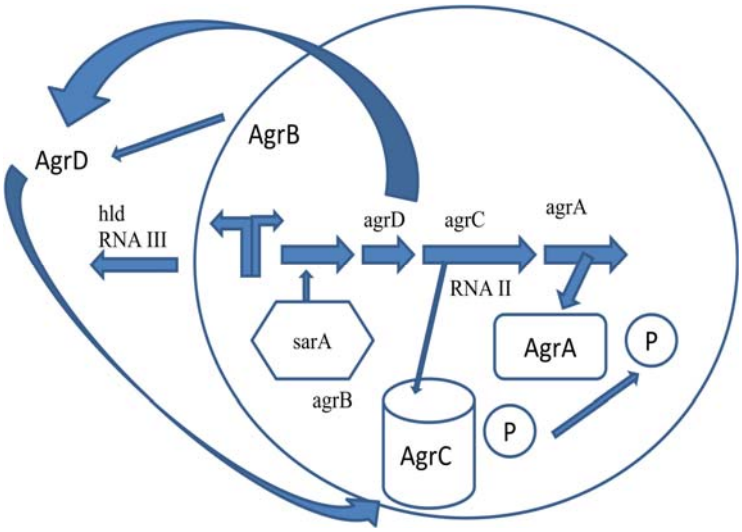


Figure 1.9 *S.aureus* accessory gene regulator and accessory regulator

The accessory gene locus consists of two transcripts: RNAII and RNAIII. RNAII encodes four genes (Agr A.B.C.D). Accessory gene regulator (Agr) transcription of the V8 gene occurs at the post-exponential phase of bacterial growth, being positively regulated by Agr (accessory gene regulator) and negatively regulated by Sar A (staphylococcal accessory regulator) **(Saenz, H. L. et al., 2000).**

1.9.1.A Aureolysin

Aureolysin is a *S. aureus* zinc metalloproteinase. The exact mechanism of aureolysin maturation is unknown; analysis of the Aur reveals that it is cleaved between Glu and an Ala residue, which is a characteristic of endopeptidase SspA and Spl proteases. Ribbon presentation of the overall structure of aureolysin is presented in **(Figure 1.10).** As mutations of SspA do not affect the activity of Aur, it is possible that Aur achieves catalytic maturation in a Spl-protease-dependent manner, this is because Spl proteases have no requirement for proteolytic maturation **(Reed.S et al., 2001).** However it is common among the thermolysin enzymes to activate themselves auto -catalytically **(Miyoshi S *et al.,* 2000).** Aureolysin has been known to cleave the plasma proteinase inhibitors, α 1-antichymo-trypsin and α 1-proteinase inhibitor **(Rapala-Kozik *et al.,* 1999).** The structure of the aureolysin gene is conserved among the *S.aureus* strains. This argues in favor of the likelihood that the enzyme may have an important housekeeping function **(Sabat *et al.,* 2000).**

Figure 1.10 Ribbon presentation of the overall structure of aureolysin

The α helices are shown in Red, the β strands are in Green, and the loops are in Yellow. The calcium ions are represented by Brown spheres and the zinc ion is represented by a Blue sphere. (Taken from protein data bank with open access permission IBQB) (**Banbula, A *et al.*, 1998).**

1.9.1. B V8 protease (SspA)

S. aureus sspA V8 protease (Endoproteinase-Glu-C) is a serine protease that specifically cleaves peptide bonds on the carboxyl side of either aspartic or glutamic acids **(Prasad *et al.*, 2004)**. This proteolytic enzyme is limited to glutamic acid in the presence of ammonium. V8 has a molecular weight of 27 KDa and pH between 4.0- 7.8. V8 protease has structural similarity with trypsin and exfoliative toxins A & B (ETA & ETB), which cause staphylococcal scalded skin syndrome in newborn and in young children **(Redpath *et al.*, 1991).** . Ribbon presentation of the overall structure of V8 protease is presented in **(Figure 1.11).**The amino terminal of the V8 protease determines its substrate specificity **(Prasad *et al.*, 2004)**. Like other extracellular proteases V8 is involved in colonization and infection of human tissue. It is required for the maturation of the *cysteine protease* staphopain B (sspB) and inactivation of sspC (an inhibitor of sspB) **(Massimi et *al.*, 2002).** Perhaps V8 is the most abundant of *S.aureus* secreted proteases which is also important for transition from adhesive to non- adhesive phenotype via degradation of fibronectin-binding proteins, and other proteins on the surface of *S.aureus* cells **(Karlsson A et al., 2001) (Mcgavin *et al.*, 1997).** V8 may also protect bacteria from the host defense by cleaving the immunoglobulin classes IgG, IgA and IgM and also deregulate host derive proteolytic activity by cleaving α1-prtease inhibitor **(Prokesova *et al.*, 1992 Randall J Brezski et al., 2010).**

Figure 1.11 Ribbon representation of the structure of V8 protease.

Protein chains are colored from N-terminal to the C-terminal using a rainbow color gradient. The two domains, built around a six-stranded antiparallel "β" sheets characteristics of the trypsin like serine proteases folds which are shown in green for domain I and blue for domain II. The "α" helix is presented here in red color and loops are shown in green and yellow colors. (Picture is taken from (RCSB) protein data bank with open access permission for 1QY6 **(Prasad *et al.*, 2004)**

1.9.1-C Staphopain A (scpA)

Staphylococcal cysteine protease staphopain A (ScpA) is secreted as a zymogen inactive (define) form and is activated by Aurolysin. . Ribbon presentation of the overall structure of staphopain A is presented in **(Figure 1.12).** ScpA is able to degrade elastin, the extracellular cystatins C, D and E/M are hydrolyzed by both staphopain A &B. The normal activity of cystatins downregulated by staphopain A, B indicating that bacterial enzymes can cause disturbance of the host protease-inhibitor balance **(Vincents *et al.*, 2007).** These proteases appear to reside outside the cascade of activation previously described, as mature staphopain A was observed in the *aur*, *sspA* and *sspB* mutant strains **(Kantyka *et al.*, 2011).** Several in vitro studies suggested these proteases are the virulence factor **(Shaw *et al.*, 2004)**. In addition, *S. aureus* proteases disturb the antimicrobial barrier by degrading adhesion junction (**Amagai *et al.*, 2000).**

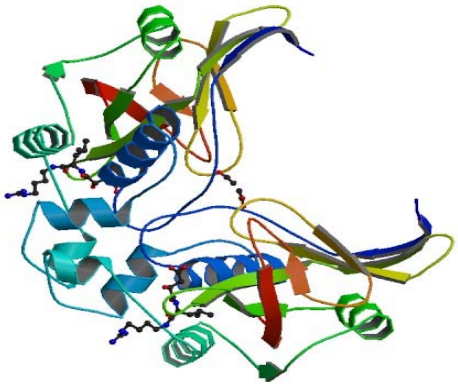


Figure 1.12 Ribbon representation of the structure of staphopain A

Staphopain A shows a typical chymotrypsin like fold. Domain I and II are shown respectively in red and green. A connecting linker is depicted in grey. The catalytic triad residues are shown in yellow (picture is taken from (RCSB) protein data bank with open access permission for 1CV8) (**Hofmann *et al.*, 1993).**

1.9.1-D Staphopain B (SSpB)

Staphopain B is a cysteine protease, having a papain like fold with molecular weight of 20 kDa

(**Figure 1.13) (Kantyka *et al*, 2011).** SSpB is translated from an operon (An operon is a functioning unit of genomic DNA containing a cluster of genes under the control of a single promoter) contiguous to the gene for the serine protease (*sspA*) **(Chan *et al.*, 1998).** Staphopain B is secreted proteolytically processed in culture supernatants; V8 protease is proposed to be responsible for this cleavage **(Kantyka *et al.*, 2011).** Staphopain B is able to degrade elastin, fibrinogen, fibronectin, and kininogen and exhibits a strong preference for substrates where arginine is preceded by a hydrophobic amino acid. This protease promotes the detachment of primary human keratinocytes from their substrates **(Massimi *et al.*, 2002)**. Along with other extracellular proteases it is involved in colonization and infection of human tissue **(Massimi *et al.*, 2002).** Prematurely activated/folded staphopain B is inhibited by staphostatin B which is probably required to protect staph cytoplasmic proteins from degradation by staphopain B. In the cytoplasm prematurely activated/folded staphopain B forms a stable complex with staphostatin B SspC, inhibiting its function **(Massimi *et al*., 2002).**

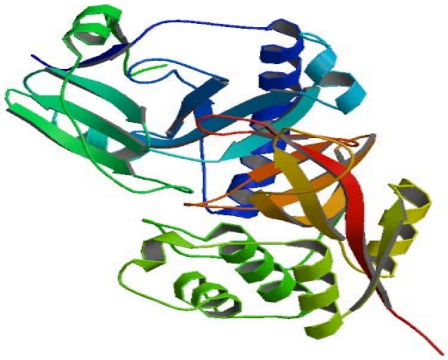


Figure 1.13 Ribbon representation of the structure of pro- staphopain B

Staphopain B shows a chymotrypsin like fold. The two domains, built around antiparallel "β" sheets characteristics of the chymotrypsin like folds which are shown in green for domain I and blue for domain II. The "α" helix is presented here in red color and loops are shown in green and yellow colors. (Picture is taken from (RCSB) protein data bank with open access permission for 1X9Y) (**Filipek,** R *et al.*, 2004).

Table 1.7 *S.aureus* extracellular proteases

Protease	Aureolysin	Staphopain A&B	V8 protease
Related gene	*Aur*	*scpA, SSpB*	*sspA*
Characteristics	Low substrate specificity cleaving peptide toward the N terminal	Staphopain A and B are the major secreted cysteine proteases	Member of glutamyl endopeptidase family of enzyme. Synthesized as a proenzyme activated by metalloprotease
Functions	Activate proV8 into active form. Modification of the bacterial cell surface protein by cleavage of N-terminal domain.	Broad substrate specificity Unclear role in pathogenesis	The degradation of the bacterial cell surface fibronectin - binding protein (FnBP)

1.9.2 *S.aureus* toxins

S. aureus depend on production of secreted and cell surface adherent virulence factors those are required for host colonization and disease production **(Kotler DP et al., 2007).** *S.aureus* virulence factors perform different functions, some toxins damage cell membrane and are called invasive. Some toxins lyse erythrocytes by causing hemolysis, but it is unlikely that hemolysis is a relevant determinant of virulence *in vivo* **(Bohach *et al.*, 1997)**. Leukocidin causes membrane damage to leukocytes, but is not hemolytic (**Marrack *et al.*, 1990).** Each of these exotoxin exhibits at least three biological properties, superantigenicity, pyrogenicity and cytotoxicity. Following are the different types of *S.aureus* toxins.

1.9.2.1 Superantigens toxins:

S.aureus secrete family of non- glycosylated low-molecular-weight exoproteins called superantigen **(Spaulding et al., 2013).** Superantigens have molecular size ranging from 19,000 to 30,000 Da and are resistant to heat, proteolysis, acids and are highly resistant to desiccation (remain biologically active after being dried on petri dishes for more than one year). *S.aureus* strains secrete at least 24 serologically distinct superantigens. Recent studies suggest that host-pathogen interactions stemming from the production of *S.aureus*- derived virulence factors, such as superantigens (entertoxin B) and alpa-toxins, contribute greatly to the skin inflammation seen in AD **(Skov L et al., 2000).**

Superantigens contains a conserved overall structure made of two major protein domains: an amino-terminal oligosaccharide/ oligonucleotide binding (O/B) fold, comprised of a

barrel and a carboxy- terminal- grasp domain made of antiparallel-strands, with domains connected by a central diagonal α- helix **(Mitchell DT et al.**, 2000**).** Based on small variations in this common core structure, *S.aureus* superantigens can be categorized into} 4 major groups. Group I include TSST-1 and TSST-ovine, SE-1 X. , Group II SEB, SEC, Group III SEA, SED, and SEE. , Group IV include SE-1K to SE-1 M, SE-1 V AND SPEH

The exoproteins produced by *S.aureus* contributes its ability to colonize and cause diseas in mammalian hosts. The superantigens interfere immune system function by eliciting strong primary response which distinguish them from peptide antigen. *S.aureus* cytotoxin act locally to kill immune cells and cell surface virulence factors provide defense to *S.aureus* against the immune system **(Spaulding *et al*, 2013).** There are nine toxins that have super antigen characteristics. A 1-Toxic shock syndrome (TSST-1) **A** 2-Enterotoxins SE- (A, B, C, D, E, G, H, I).

1.9.2.1 A-Toxic shock syndrome toxin (TSST-1).

TSST-1 is expressed systemically and is the cause of toxic shock syndrome (TSS) **(Patrick. M *et al.*, 2000).** TSST-1 is weakly related to enterotoxin but does not have emetic activity; TSST-1 is responsible for 75% of TSS, including all menstrual cases **(Kotler DP et al., 2007).** The reason for the high association of TSST-1 with menstrual toxic shock syndrome is not completely clear, but it likely depends on at least three factors. (1) TSST-1 is produced in high concentration relative to the majority of other superantigens (2) TSST-1 has greater mucosal surface-penetrating ability than other superantigens **(Schlievert PM et al., 2000).** (3) Large numbers of mucosal *S.aureus*

strains produce TSST-1(**Kuehnert MJ et al., 2006**). In addition to these properties, these toxins have superantigen activity**.** TSST-1 induces the production of proinflammatory chemokines interlukin-8 (IL-8) (CCL8) and MIP-3-(CCL20) in human vaginal epithelial cells **(Peterson M et al., 2005).** These chemokines attract neutrophils and other immune cells including T cells and macrophages, to infection sites**.** This property enables them to stimulate T-cells non- specifically, without normal antigen recognition, and at 1:5 ratios whereas only 1 in 10,000 cells are stimulated during normal antigen presentation **(Davis CC et al., 2003).**

1.9.2.1 B- Enterotoxins (SE)

There are eight antigenic types named SE (A, B, C, D, E, G, H, I), *S.aureus* superantigen SEs including SEA are emetic, cause episodes of retching, vomiting, and diarrhea within 24-48-hours after ingestion of nanogram quantities and without fever (**Le Loir Y et al., 2003).** *S.aureus* SEs are the primary cause of toxin-mediated food borne illness and second leading cause of food-borne illness, their systemic effects can also cause toxic shock syndrome. *S.aureus* SEs cause prolification of T-lymphocytes and food poisoning **(Spauldling et al 2013).**

1.9.2.2 Disease causing toxin

1.9.2.2 A Exfoliative toxins (ET)

ETS are extremely specific serine proteases cleave epidermal desmosomal protein and also called as epidermlytic toxins. ETs (A, B) are associated with scalded skin syndrome (SSS) and bullous impetigo **(Amagai M et al, 2000).** Their epidermolytic effect is by possessing an

atypical glutamic acid-specific trypsin-like serine protease activity (**Ladhani et al 1999).** ETS causes separation within the epidermal, stratum granulosum and stratum corneum layers. The separation is through the stratum granulosum of the epidermis. *S.aureus* ETA has been shown to specifically cleave desmosomal desmoglein 1 **(Ladhani *et al.*, 2003).** Koji Nishifuji has suggested that the removal of amino-terminal extracellular domains of Dsg 1 by ETs is sufficient to initiate epidermal blister formation in bullous impetigo and SSSS **(Nishifuji, K., et al. (2010).**

1.9.2.2 B-Cytolytic toxins (hemolysin)

S.aureus secreted cytotxins are capable of damaging the host cell plasma membrane. *S.aureus* cytotoxin which damage the erythrocyte membrane are called hemolysins (α, β, ɣ hemolysis) and which damage the leukocyte membrane are called leucocidin (panton-valentine leukocidin -PVL). *S.aureus* cytotoxins make pores in the host cell plasma membrane and the pore formation initiated when toxin monomer binds to the cell surface and makes transmembrane channel **(Verdon et al., 2009).** The α hemolysin is the most characterized virulence factor of *S.aureus*, intially forms prepore in the cell membrane, which transitions to a mature β-barrel transmembrane pore. Panton-valentine leukocidin does not make prepore instead insert itself into the host's plasma membrane and forms a pore in the leukocyte (**Vandenesch** et al, 2012).

1.9.3-Adherence factors

Attachment of the *S.aureus* to the cell surface is mediated by several adhesions, which are following.

1.9.3.1 Surface proteins

S.aureus protein A (SPA), fibronectin binding protein A and B (FnbpA and Fnbp B), collagen binding protein, and clumping factor (Clf) A and B

A- Protein A (SPA), B- Fibronectin binding protein A and B (FnbpA and Fnbp B) C- Collagen binding protein, D- Clumping factor (Clf) A and B

PVL: Panton–Valentine leukocidin

1.9.4 Toxins relevance and link to AD

S.aureus- derived virulence factors, such as superantigen and alpha-toxin, contribute greatly to the skin inflammation seen in AD **(Skov L et al., 2000).** The enhanced allergen penetration through damaged epidermis accompanied by increased production of thymic stromal lymphopoeitin by keratinocyte (Th2-type milieu) could provide a critical link between barrier defects in AD patients with filaggrin gene mutation. Loss of filaggrin has been linked to enhanced antigen penetration into the skin and increase *S.aureus* and viral growth in the skin as well as susceptibility to the cytotoxic effects of *S.aureus* alpha-toxin **(Miajlovic H et al., 2010).**

The severity of AD correlates with the number of superantigen-secreting *S.aureus* organism colonizing the skin **(Boguniewicz M et al., 2010).** The superantigen also play role in the exacerbation of AD. A murine model of skin inflammation has shown that *S.aureus* superantigens plus allergens have an additive effect in driving cutaneous inflammation **(Ong.P. Y et al., 2008**) High levels of IgE antibodies in AD patients against superantigen found on the skin correlates with AD severity **(Bunikowski R et al.,**

1999). The superantigen SEB applied to the skin can induce eczematoid skin changes and T regulatory cells lose their immunosuppressive activity. Suggesting a novel mechanism by which superantigens could augment T cell activation in patients with AD (**Ou LS et al., 2004)**. Superantigen induces T cell expression via stimulation of IL-12 production. Superantigen also induces corticosteroid resistance and an increase in AD severity. Superantigens selectively induce T cells to secrete IL-31, a highly pruritogenic cytokine that regulates filaggrin expression and is produced by Th2 cells (**Cornelissen C et al., 2012).**

1.10 Antimicrobial peptides (AMP)

Antimicrobial peptides (AMP) are also called host defense peptides; these peptides are a conserved component of the innate immune response and are found among all classes of life **(Jenssen *et al.*, 2006)**. The characteristics of antimicrobial peptides are presented in the **(Table 1.8).** Structurally there are five different subgroups of AMP**. First subgroup** contains human dermcidin a small anionic peptide present in the surfactant extracts, broncho-alveolar lavage (BAL) fluid and epithelial cells. They require Zn as a cofactor for their antimicrobial activity. Anionic AMPs are active against both gram positive and gram negative bacteria **(Schittek, B et al., 2001).** A **second subgroup** contains LL37 a linear α helical short cationic peptide having < 40 amino acids, these linear cationic peptides also lack cystein residues and sometime have a hinge and kink in the middle **(Gennaro, R et al., 2000). Third subgroup** contains histatins which is a cationic peptide and rich in proline, histidine and arginine. These peptides lack cysteine residues and are linear **(Kavanagh, K et al., 2004).** A **fourth subgroup** contains anionic and cationic peptide having cysteine residue and form stable

disulphide bonds. This subgroup includes diverse family of α, β, θ defensins, found in mammals and is classified according to their peptide structure **(Ganz et al., 2002)**. A rhesus (θ) defensin is a18 residue peptide that forms a circular molecule that is crosslink by three disulphide bonds **(Weiss, T. M et al., 2002).** RTD (Rhesus theta defensin) θ defensins found in rhesus (Monkeys) has circular structure without free N or C terminus. The pattern of disulphide pairing of the θ defensins is different than α & β defensins **(Tang *et al.*, 1999). Fifth subgroup** contains anionic and cationic peptides which are fragments of larger proteins like human hemoglobin and lysozyme. These fragments are similar in composition and structure to the antimicrobial peptide of the fourth subgroup, having antimicrobial activity. However their role in innate immunity is not clear yet.

Table 1.8 the characteristics of antimicrobial peptides

Size	AMP size Varies from 6 – 59 amino acids
Sequence	AMP Contains basic amino acids residue lysine or arginine. Some peptides may have amino acid repeat.
Charge	Anionic AMPs are rich in aspartate and glutamate, cationic AMPs are rich in arginine and lysine
Conformation and structure	AMP can assume a variety of secondary structures including α helices, relaxed coils and antiparallel β sheets structures.
Hydrophobicity	This characteristics makes AMP water-soluble and makes partition into the membrane lipid bilayer.
Amphipathicity	AMP contains hydrophilic amino acids aligned along one side and hydrophobic amino acid residues aligned along the opposite side of the helical molecule.

Commented [DJD1]: Again, bits like this need to be a table or written properly.

1.10.2 Human Defensins:

α and β defensins are found in humans and are classified according to their peptide structure. Biological image assemblies for defensin have been described in **(Figure 1.14)** originally defensins were isolated from human leukocytes, and then it was found that defensins are also produced by epithelial and mucosal cells **(Hallock *et al.*, 2003).** Defensin genes are located in the cluster of loci on chromosomes 8p 22-23. Defensins are amphipathic compounds having 12-50 amino acids, cysteine rich peptide with size range of 3-6 kDa and having positive charge range of +1 to +11. These peptides are important mediators of innate immunity and potent broad spectrum antibiotics which can be used as potential novel therapeutic agents (**Boman, *et al.*, 1995).**

Defensins can exist in multiple states (Monomers, dimers etc) in vivo, depend upon factors such as, presence or absence of pathogen, inflammation and pH. The crystal structure of α defensins revealed that it has six cysteines with three disulfide briges located in a 1-6, 2-4, 3-5 pattern and exists as a dimer in solution, To date, six alpha-defensins have been identified in humans. Four of these, designated human Neutrophil peptides (HNP) 1,2,3, and 4 the remaining two , human defensins 5 and 6 are expressed in intestinal paneth cells. Small intestine paneth cells secrete very high concentration of α defensins which contribute to innate defense of GI mucosal surface **(Ganz *et al.*, 1985).** β defensins were isolated from the bovine tracheal epithelial cells in 1991 and are found in human. Epithelial cells secrete defensins continuously and sometimes in response to infection, IL1-β and LPS **(Mathews *et al.*, 1999).** Normally the plasma level of α defensins is in the range of 40 to 200 ng/mL but that level can be reach upto 10 -100

µg/mL during sepsis **(Ayabe *et al.*, 2000).** When neutrophils ingest microorganisms they are exposed to highly concentrated HNP in phagocytic vacuoles contributing the key non-oxidative killing mechanism of neutrophils **(Faurschou *et al.*, 2003)**.

Beta defensins are rich with β sheet stabilized by disulfide bridges, having six cysteines, which are located in a 1-5, 2-4, 3-6-pattern **(Lehrer *et al*., 2002).** β defensin including six cysteins with three intramolecular disulphide bonds **(Schutte, B. C et al., 2002)**. β defensins were isolated from the bovine tracheal epithelial cells in 1991 and are found in human. β –defensins are expressed by epithelial cells of skin, gastro-intestinal tract, kidney, respiratory tract, eyes, mammary glands, male and female genital tracts **(** **Com *et al*., 2003)**. Epithelial cells secrete defensins continuously and sometimes in response to infection, IL1-β and LPS **(Mathews *et al.*, 1999).**

Beta defensins are salt sensitive except beta defensin 3 which is salt insensitive. Previously it was reported that shape and charge distribution of the hBD2 monomer are similar to those of other defensins, an additional alpha-helical region makes this protein topologically distinct from the mammalian alpha and beta – defensin structures **(Oren *et al.*, 2003).** The hBD1 and hBD2 are monomers in solution while hBD3 can exit as a dimer; these structures provide the first detailed description of dimerization of beta-defensin. The quaternary octameric arrangement of hBD2 is conserved in the crystal form. **(Hoover *et al.*, 2000)**. α and β defensins cysteines number 5 and 6 are adjacent, cysteine connectivity pattern cannot alter the structure The positive charge of defensins attributes the important action against bacteria, fungal and parasites. Loss of positive charge resulting in loss of antibacterial, antifungal and antiparasites activity

(Lichtenstein *et al.*, 1991). (Ganz and Weiss, 1997); Sawai *et al.*, 2001). The modes of action by which AMP kill bacteria is varied and includes disrupting membranes, interfering with metabolism, and targeting cytoplasmic components **(Wildman *et al.*, 2003).**

A fundamental difference between prokaryotic and eukaryotic cells is the higher proportion of anionic phosphtidylglycerol (PG) and cardiolipin (CL). Presence of higher proportion of PG and CL in the bacterial cytoplasmic membranes makes it more electronegative, while extremely low level or absent in the mammalian membranes **(Koppleman *et al.*, 2001)?** Although at present, AMP are believed to exert electrostatic interaction primarily on bacterial membranes, new evidence is suggesting that AMP activity might be broader, including selective inhibition of intracellular targets **(Cudic.M et al., 2003)**.

1.10.3 Important of AMPs in humans

AMPs are widely distributed throughout nature and have been discovered in certain bacteria, protozoa, fungi, plants and multicellular animals. Human AMPs protects epithelia against invading microorganism and assisting neutrophil and platelets (Peschel et al., 2002). Important functions of antimicrobial peptides are described in **(Table 1.9)** AMP has been demonstrated to kill gram+ve and gram-ve bacteria's, enveloped viruses, fungi and even cancerous cells **(Hoskin *et al.*, 2008).**

Defensins have antiviral activity against envelope viruses such as HSV virus and HIV virus **(Chang *et al.*, 2005).** AMPs are known to be multi-factorial having a role as a mediator of inflammation effects on epithelial and inflammatory cells. AMP also has

the ability to modulate inflammation and immunity by functioning as immunomodulators **(Hunter *et al*., 2002).** These roles have impact on diverse process such as proliferation, immune reduction; wound healing, cytokines release, chemotaxes and redox homeostasis (**LIU, L et al**., **2003).**

AMPs are chemotactic for human monocytes, dendritic cells, PMNL and T-cells, and have mitogenic effects on murine fibroblast and epithelial cells **(Yang *et al.,* 2000)** Defensins act to mobilize and activate various leukocytes for innate and adaptive immune response **(Soruri *et al.,* 2007).**

The target of AMPs that underlies their specificity for microbes is the difference in lipid composition between the membranes that surround microbes and cells in our body. Microbes have plasma membranes that contain phospholipid with negatively charged head groups on the outer leaflet, the side exposed to the outside. Resistanc to these AMPs, in general, is difficult to develop because it requires redesign of membrane lipid composition and topology (**Perron GG et al., 2006**

Table 1.9 important function of antimicrobial peptides

Antimicrobial peptides (AMPs)	**Important functions**
Defensins,indolicidine and LL37	Broad-spectrum antibacterial
Defensins NP-1 and NP-5	Synergy with other peptides
Protegrin, indolicidine and histatins	Antifungal
LL-37	Anti-endotoxin
Indolicidin, protegrin and defensins	Anti-enveloped virus (HIV, HSV, VSV)
Indolicidin and defensins	Anticancer
PR39 and defensins	Wound healing
Indolicin and defensins	Antiparasite

1.10.4 Antimicrobial peptides and biotechnology

AMP and their synthetic derivatives defend host organism against microbes having antimicrobial and immunomodulator activities. The crystal structure of defensin is presented in **(Figure 1.14).** Multi drug resistant bacteria are a severe threat to public health. Conventional antibiotics are becoming increasingly ineffective as a result of resistance, and it is important to find new antibacterial strategies.. AMPs do not induce microbial resistance, which makes them extremely valuable in the development of new treatment for multi-drug resistant infection (**Afacan NJ et al., 2012)**. Having cell membrane- lytic effect AMP can kill tumor cell, and some cationic AMPs (CAPs) can trigger apoptosis in cancer cells via mitochondrial membrane disruption. Furthermore, certain CAPs are potent inhibitors of blood vessel development (angiogenesis) that is associated with tumor progression (**Mader, J. S et al., 2006).** Host defense peptides and their synthetic innate defense regulator (IDR) peptide derivatives are being investigated for their potential value in the therapy and prophylaxis of infection. Hancock RE group developed a synthetic cationic peptide-IDR-1002 which enhanced chemokine induction activity in vitro correlated with stronger protective activity in vivo in the *S.aureus* invasive infection model and also afforded protection against gram-negative bacterial pathogen Escherichia coli (**Nijnik A et al., 2010)**. Defensins have immunomodulator effect by modulating the immune response in addition to antimicrobial action. Pro-inflammatory role of defensins both in epithelial and immune cells are called alarmin molecule or damage associated molecule. However at higher concentration cytotoxic effects against various eukaryotic cells and tumor cells have been described (**Lichtenstein et al., 1991).** In addition, defensins exhibit pro inflammatory properties

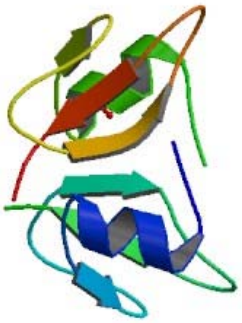


Figure 1.14 Biological image assemblies for Defensin

Where protein chains are colored from N-terminal to the C-terminal using rainbow color gradient. Red color potentially disordered region and Blue color probably ordered region. Hydropathy has been calculated using a sliding window of 15 residues and summing up scores from standard hydrophobicity tables. Red is hydrophobic and Blue is hydrophilic (picture is taken from (RCSB) protein data base with open access permission for1FD3).**(Hoover*etal.*,2000).**http://www.rcsb.org/pdb/explore/images.do?structureId=1FD3#

1.11 Summary

S. aureus is considered an environmental factor in the pathogenesis of AD. *S.aureus* is not a member of the normal skin micro flora, although 90% of individuals with AD are carriers in their nasal and perineal areas of the skin. *S. aur*eus can secrete proteases including serine V8, cysteine staphopain A-B and aur (metallo) proteases **(Shaw *et al*., 2004)**. Several *in vitro* studies have suggested these proteases are key virulence factors in AD (**Shaw *et al*., 2004)**

Protease and protease inhibitor balance plays a significant role in the maintenance of barrier function in AD. Part of skin defense against *S.aureus* exoproteases is an antiproteolytic balance which can be maintained by the defensin family Elafin, SLPI and Cathelicidin **(Wiedow *et al.*, 1990).** Unfortunately many of these antiprotease peptides are substrates for an *S.aureus* exoproteases and therefore are readily degraded, e.g. human extracellular cysteine protease inhibitors are degraded by staphylococcal secreted cysteine proteases, staphopain A **(Vincents *et al.*, 2007)**. Some atopic patients have decreased expression of Cystatin A at their lesional epidermis which could be associated with a reduced inhibition of both endogenous and exogenous cysteine proteases **(Cheng *et al.*, 2009).** Transgenic mice carrying a null mutation in the gene encoding either cystatin M or cystatin E display severe barrier abnormalities, affecting cornification and desquamation, and die shortly after birth **(Zeeuwen PL *et al*., 2002).**

S.aureus infection and disrupted antimicrobial defense system is a common problem in AD. Altered level of antimicrobial peptide has been reported in AD skin that

could be linked to impaired barrier function found in atopic dermatitis patients **(Clausen *et al.*, 2013).** Antiprotease action of AMP along with antimicrobial action can protect skin barrier from damaging effect of *S.aureus* secreted proteases. *S. aureus* proteases aggravate antimicrobial barrier by degrading adhesion junction (Amagai ***al*., 2000).** Antimicrobial barrier (AMB) and permeability barrier are linked together because both water loss and pathogen invasion occurs by the breakdown of intercellular junction present in the lipid matrix **(Borkowski *et al.*, 2011).**

1.12 Hypothesis:

The novel antimicrobial peptide human beta defensin-2 protects the skin barrier from proteases secreted from opportunistic skin pathogens associated with Atopic Dermatitis

1.12 A Aim: To study the novel role for hBD2 in protecting skin barrier from damaging effect of *S .aureus* secreted proteases by having both antimicrobial and antiprotease action in relation to disease severity in AD.

1.12 B Objectives:

1-To confirm *S.aureus* secreted V8 protease can damage the HaCaT cell monolayer.

2- To determine the antiprotease action of hBD2 by protecting HaCaT cell monolayer from *S.aureus* secreted V8 protease

3- Protection of the HaCaT cell monolayer from *S.aureus* secreted V8 protease by expressing hBD2 with external factors**.**

Chapter-2 2- Materials and Methods

2.1 Materials

Primary antibodies were purchased from Invitrogen (Paisley UK). These were Rabbit anti human Claudin-1, and Rabbit anti human ZO (Invitrogen), mouse anti human desmoglein (AbD SeroTec). Goat anti hBD2 primary antibody Pepro Tec (NJ, USA); secondary antibodies; anti Rabbit and anti-mouse secondary antibody (Licor Biosciences UK); Alexa Fluor 568 Goat anti-Rabbit IgG antibody (Thermo Fisher), DEFB4 siRNA from applied biosciences and Lipofectamine 2000 transfection reagent from Invitrogen. DEFB4 plasmid OriGene (Rockville,MD) cat#C219487 (USA), *S.aureus* strain 8325-4 , RN6390, SH1000 including *S. Epidermidis*, mutant of the parent strain 8325-4, LES17(L17) sspB pAUL-A ssPB; LES22 (L 22) sspA: Paz106 sspABC, LES27 (L27) scpA: Paz106 scpA, Staphopain B from Bio Centrum (Krakow Poland); V8 and Trypsin from Worthington Biochemical Corporation (Lakewood, NJ USA), Leupeptin (Sigma–Aldrich), Z-Phe-Arg-7-amido-4 methylecoumarine substrate Calbiochem (CA, USA), Recombinant Human beta defensin Cayman chemical (MI, USA), SDS-12% polyacrylamide gel containing 0.1 % β-casein (Sigma, St.Louis,MO.), LDH assay kit (Clontech,CA,USA), Bicinchoninic Acid assay kit (Bio-Rad, USA), Recombinant human IL-1β and human BD2 ELISA development kit from Peprotech (NJ, USA), ABTS enhancer (Sigma –Aldrich), Transwells from Corning incorporated life science (MA,USA), FITC-albumin; Lucifer Yellow (Sigma–Aldrich), *S.aureus* LTA (Sigma–Aldrich). Taq man Reverse Transcription Reagent kit (Applied Biosystems), Taq man probes and primers were purchased from Applied Biosystems(Warrington-USA). Neupage LDS sample buffer (catalog# NP0007) (Lifetechnology), Novex

zymogramRenaturingBuffer(Lifetechnology), DEFB4 plasmid was from OriGene, cat. no. RC219487**Table 2.1 primary and secondary antibodies**

Primary antibodies	**Dilution**	**Catalog #**	**Secondary antibodies**	**Dilution**	**Catalog #**
Rabbit-antihuman Claudin-1	**1/500**	**Invitrogen # 51-9000**	Goat anti-Rabbit IRDYE 800CW	**1/5000**	Li-Cor Biosciences UK #926-32211
Rabbit-antihuman ZO-1	**1/200**	**Invitrogen #40-2200**	Goat anti-Rabbit IRDYE 800CW	**1/5000**	Li-Cor Biosciences#926-32211
Mouse-antihuman desmoglein-1	**1/100**	**AbD SeroTec# MCA 2271T**	Goatanti-Mouse IRDYE 680LT	**1/5000**	Li-Cor Biosciences #926-68020
Goat-antihBD2	**1/250**	**Pepro Tec#500-P161**	Alexa-Fluor568-Goat-anti-Rabbit-IgG	**1/1000**	Thermo Fisher #A11011

			antibody		

2.1 A Western blotting materials

M-PER Lysing buffer from (Thermoscientific); BCA reagents; Novex 4-12 % gradient gel by Invitrogen. Precision plus Protein Standards Marker from Bio Rad (CA, USA), Bench Mark Pre-stained Protein Ladder from Invitrogen (UK).

2.1 B- Cell and tissue cultures:

The immortalized human keratinocyte cell line (HaCaT) cells was orignally (a generous gift of prof. Dr. N. Fusenig, Heidelberg, Germany) **(Boukamp et al, 1988).** The HaCaT was kindly provided by a colleague Siao Pei Tan as frozen cultures in cryovials at as low a passage number of 20.

hBD2- hBD3 over-expressing HaCaT cell lines, constructed by Dr Brian McHugh (a senior post-doctoral researcher in the Davidson Group at the University of Edinburgh / MRC Centre for Inflammation Research) using shRNA transfection to HaCaT cells.

2.2 Methods

2.2.1 Recovering cells from liquid nitrogen

To recover the cells the sample were rapidly warmed to 37°C in a water bath with gentle agitation immediately following removal from liquid nitrogen. Then they were

placed into 25mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum and (Penicillin 100 IU/mL + Streptomycin 10 µg/mL). Cell cultures were kept in an incubator at 95% relative humidity with 5% CO2 at 37°C. Cells were passaged using 0.05% trypsin and 0.02% EDTA/100 mL (T/E) to detach and disperse adherent cells. Then excess trypsin was quenched with DMEM + fetal calf serum, and then gives a quick wash by spinning the cells at 300 x g for 5 minutes. The old medium was removed before adding fresh 1ml of the DMEM + serum into the pellet of all the samples along with gentle pipetting dispersed the cells. The cells were counted by using nucleocassette from Chemometec and then seeded at a density of 4 $x10^5$ cells /mL of media in a 12 well plates and 2 $x10^5$ cells /350µL of DMEM medium on the transwell insert. After 2-3 days of culture when monolayer was approx 90% confluent, DMEM + serum medium was replaced with Gibco keratinocyte serum free medium (G-SFM) before performing the experiment.

2.2.2 Collection of supernatant from different *S.aureus* strains.

S.aureus strain 8325-4, RN6390, SH1000 including *S.Epidermidis* kindly provided by Dr Fitzgerald Department of Microbiology Royal SVM Dik Vet college Edinburgh university, cultured in 10 mL of Tryptic Soy Broth (TSB) without antibiotic while mutant strains LES17(L17); LES22 (L 22) and LES27 (L27) cultured in TSB broth with addition of 5µg/mL Erythromycin. All the strains cultured to late stationary phase in a shaker incubator at 200 rpm for 24 hours at 37°C. The concentration of bacteria in a suspension was measured by calculating? OD (optical density) in a spectrophotometer. The optical density was determined at a particular wavelength in the range of (420-660

nm) which correlated with different growth phases. Generally cells were in their late stationary phase of growth and OD was in the range of 600. Bacteria free conditioned media was collected by centrifugation at 5000 x **g** before filtering through a 0.2 µm filter to remove any cellular material. Optical density (OD) was measured at 595 nm to determine cell density where an OD 595 of 1:0 corresponded to $1x10^9$ colony forming units (CFU/mL).

2.2.2 A Trypan Blue Permeability

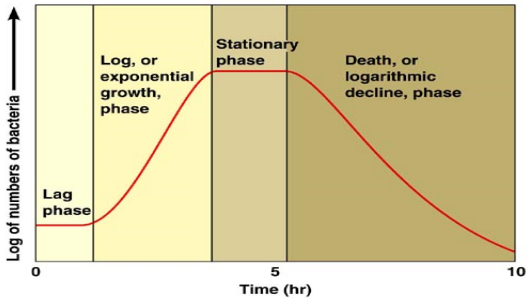
Trypan blue is a viable cell exclusion dye that selectively permeates through compromised membrane of dying cells. It is based on the principle that live cells possess intact cell membranes that excluded trypan blue. Typically 0.1% Trypan blue solution was used at a 1:4 dilution.

2.2.2 B LDH assay for cells cytotoxicity

HaCaT cells were exposed to *S. aureus* RN6390 CM for 30-120 min, in either the presence or absence of leupeptin. 100% permeability of cells was obtained by adding 0.1 % Tween-20. All assays were performed in triplicate. The LDH release assay was performed using LDH cytotoxicity detection kit in 96 well plates before measuring absorbance at 490 nm in an ELISA plate reader. To determine the cell-mediated cytotoxicity we calculated the average absorbance of triplicate samples and controls, after correcting for background.

2.2.3 Heat killed *S.Epidermidis* and their fractionates

S. Epidermidis cultured in 10 mL of TSB broth without antibiotic in a shaker incubator at 200 rpm for 24 h at 37°C. Centrifuge the media for 10 min at 5000 x g to make a cell pellet then discard the conditioned media and washed the pellet twice with PBS before suspended the cells in PBS. The optical density was determined at a particular wavelength in the range of (420-660 nm) which correlated with different growth phases. Generally cells were in their late stationary phase of growth and OD was in the range of 600nm. Supernatant was collected and filtered through a 0.2µm filter to make it cell free.



During the lag phase bacterium adapt to the new environment and the growth is very low, which is almost close to zero. The log phase is the period of optimal growth, and microbes approach the upper limit of their continued growth called carrying capacity (k). The stationary phase is the point at which, bacterial population level out and population growth is near to zero again and this stage may last for a long period of time. Ultimately, waste and dead cells begin to accumulate, causing the death phase. Optical density (OD) was measured at 595 nm to determine cell density where an OD 595 of 1:0 corresponded to $1x10^9$ colony forming units (CFU/mL). After measuring OD, the cells were re-

suspended in PBS and heated at 100 °C for 30 mint. The effectiveness of killing by heat was tested when I spread 100 µL of heat killed *S.Epidermidis* CM on the Agar plate and leave in the incubator at 37°C over night. There was no *S.Epidermidis* growth seen on the Agar plate.

2.2.4 Zymographic assay with supernatant from *S.aureus* strains

Zymographic analysis of the proteases present in the supernatant collected from all the strains. The exoproteins present in the C.M of *S.aureus* strain 8325-4, RN6390, SH1000 were concentrated by rotary evaporator for 2 hours. Protease activity associated with serine protease (sspA) was detected using zymographic analysis described by **(Arvidson et al., 2000).** 52.5 µL vol of each sample was mixed with 17.5 µL of 4X Neupage LDS sample buffer without adding β mercaptoethanol and no heating, then left it at room temperature for 15 minutes. Samples were loaded on an SDS-12% polyacrylamide gel containing 0.1 % β-casein (Sigma, St.Louis,MO.), electrophoresis was performed with a constant voltage of 140 V using SDS buffer precooled at 4°C. Proteins were electrophoresed under standard conditions. After electrophoresis, the gel was renatured with Novex zymogram Renaturing Buffer for 30 min then decanted the buffer solution. The gel was developed by placing in developing buffer overnight. Next day the gel was washed with deionized water, and then stained with 0.5 % coomassie blue for 1-2 hours with gentle agitation at 37°C. Zones of hydrolysis were visualized after destaining gel in Coomassie destains solution or deionized water -2-3 hours.

2.2.5 DEFB4 (hBD2) siRNA knock down assay

HaCaT cells seeded at a density of 5 x10^5 cells /250 µL of plain DMEM without any additive in a 6 well plate.

Preparation of Lipofectamine (transfection reagent) coated control siRNA and DEFB4 siRNA

Before starting experiments I made two groups having twelve tubes in each group.

Group A

-In the first batch of 6 tubes added 5 µL of control siRNA into 250 µL of plain DMEM

- In the second batch of 6 tubes added 5 µL of Lipofectamine (Lipofectamine 2000 is a common transfection reagent, produced and sold by Invitrogen, used in molecular and cellular biology) into 250 µL of plain DMEM

Mix both batches together and tubes were left at room temperature at least thirty minutes. During that period Lipofectamine coated over the siRNA .

Group B

- In the first batch of 6 tubes added 5 µL DEFB4 siRNA into 250 µL of plain DMEM

- In the second batch of 6 tubes added 5 µL of Lipofectamine into 250 µL of plain DMEM

The tubes in both batches were mixed together and left at room temperature at least thirty minutes, during that period Lipofectamine particles coated over the DEFB4 siRNA particles. The content of the tubes in the group A and B were finally poured into the two six wells plates contained non-confluent HaCaT cells. Lipofectamine coated control and DEFB4 siRNA belong to group A and B were poured separately into six wells plates contained non- confluent HaCaT cells. The transfected cells in the six wells plates were kept in the incubator for 12 hours. Next day old medium was replaced with regular DMEM + Penicillin & Streptomycin + serum. After 2 days of transfection, HaCaT cells were ready to use in the experiments.

2.2.6 HaCaT cell transduction with DEFB4 plasmid to overexpress hBD2

HaCaT cells were grown in six-well plates to 30-50% confluence in plain DMEM without any additive. After addition of condition medium cells were infected with lentivirus taged with DEFB4 plasmid and incubated at 37 ℃ overnight. The next day medium was replaced with serum plus medium. After expansion in culture for 48-72 h HaCaT cells were collected and analyzed for hBD2 expression.

Commented [aq2]:

2.2.7 Enzyme kinetics

The following buffer was used for enzyme kinetics both for *S.aureus* V8 and Staphopain B, 4mM EDTA, 10mM DTT, and 400 mM sodium acetate (pH 7.8). *S.aureus* serine protease V8 (500ng in 20 µL of DH2O) was preincubated with 60 µL of buffer for 15 min at 37°C before adding (20 nM in 20 µL of DH2O) final concentrations in the assay of a quenched fluorescent substrate Z-Phe-Arg-7-amido-4-methylcoumarin (1µM) stock

concentration. Substrate hydrolysis was monitored continuously by emission of fluorescence using a spectrofluorometer at 360/20 nm excitation and 460/20 nm emission wavelength. One unit was defined as the amount of enzyme that will hydrolyze 20 nM of Z-Phe-Arg-7-amido-4-methyl coumarin per min at 37°C with pH 7.8. *S. aureus* proteases staphopain B and V8 interaction with recombinant hBD2- synthetic hBD2- (C-S) - cysteine replace with serine- (SCB) scrambled peptide and LL37 was assayed spectrofluorometrically. In addition, supernatant obtained from RN6390 and 8325 *S.aureus* parent strain were similarly assessed for the hydrolysis of Z- Phe-Arg.

2.2.8 Protein extraction, electrophoresis, and Western blot analysis

For experiments, HaCaT cells were typically seeded at a density of 4 $x10^5$ cells/cm^2 in 12 wells plate. Confluent HaCaT cell monolayer in serum free medium (SFM) was exposed with V8 protease for 24 hours. Cells were lysed with M-PER Lysing buffer (A reagent extract cytoplasmic and nuclear protein from cultured cells, utilizes a proprietary detergent in 25mM bicine buffer pH 7.6). The lysates were homogenized by spun down at 13000g for 10 minutes. The protein concentration of both the treated and untreated samples were determined using a Bicinchoninic Acid (BCA) assay kit according to the manufacturer's instruction.

Purified Protein samples were mixed with loading buffer and 5 µL of each sample was electrophoresed on a Novex 4-12 % gradient gel. The proteins were transferred to Immobilon-P PVDF membranes (Millipore) or Nitrocellulose blotting membrane using a protean II device (Bio-Rad Laboratories, Hercules, CA). (Nitrocellulose membrane is recommended for Licore Odessy to get maximum performance) Membranes were

blocked overnight at 4°C with 5% (w/v) dried skim milk in PBS/ 0.05 % Tween-20 (PBST).

Primary antibodies were diluted in 5 % milk in PBST and incubated with the membrane at room temperature for 2 hours. Membranes were rinsed three times in PBST and washed with agitation for 15 min followed by 2 washes for 5 min. Transblot membrane was incubated with Claudin-1, ZO-1 primary antibodies at (1:500) dilution, and Desmoglein at (1: 100) dilution. Transblots were left rolling overnight in the cold room at 4°C. Membranes were washed for 3x with PBS/0.1% Tween then incubated with Licor Odyssy anti Rabbit and anti mouse secondary antibody at (1: 5000) dilution for 30 minutes at room temperature. After three more washes with PBST, immunoreactive protein bands were detected by using Licor Odyssy according to the manufacture's instruction. Near infrared fluorescence (Licor Odyssy) with the use of fluorescent, IRDye secondary antibodies, provide sensitivity better than chemiluminescence having low background, multiplexed detection and the sensitivity of any fluorescent system. Following fluorescent dyes are appropriate for use with the Odyssey, Cy5-700, Alexa fluor 647-680, IRDye 700-800

2.2.9 Immunohistochemistry, Confocal microscopy

Immunohistochemistry assay was performed where HaCaT cells were seeded at a density of 2 x10^5 cells/cm^2 in an eight wells (0.8 cm^2 / well) chamber slides.

Immunohistochemistry step1-General procedure 2-Fixation 3-Antigen retrieval 4-permeabilization 5-Blocking and incubation 6-Staining

When HaCaT cells become 90 % confluent they were fixed with 4% Formaldehyde for 20-30 minutes and subsequently washed with PBS. Cell membrane nonspecific Fc receptors were blocked with 1% FCS for 30 minutes at room temperature. Fc receptors (Fragment, crystallizable) are a protein found on the surface of certain cells that contribute to the protective functions of the immune system. There are several different types of Fc receptors, which are classified based on the type of antibody that they recognize) Followed by washing with PBS incubated with (Rabbit anti human Claudin-1 and Rabbit anti human ZO-1) primary antibodies with concentration of (1: 200) in 0.3 % BSA then store in the dark at +4°C for overnight. Cells were washed again and incubated with alexa Fluor 568 Goat anti-Rabbit antibody with concentration of 1/400 (1/300) in the FCS and leave it in the dark at +4°C for 2-3 h. Nuclei were counterstained using DAPI nuclear stain. Phalloidin binds specifically and with high affinity to the polymerized form of actin (F-actin). Actin staining is very useful in determining the structure and function of the cytoskeleton in living and fixed cells. HaCaT cells fixed monolayer was stained with Phalloidin for 20-30 minutes and then washed with PBS and leave the cells in 500 µL of PBS.

2.2.10 IL1β/LTA stimulated HaCaT cells express hBD2

HaCaT cells seeded in 6 well chamber plates were grown to confluence then stimulated with IL1β/LTA (8

-100 ng/mL and 5µg/mL) for 24 hours. Stimulated and unstimulated monolayers were fixed with 4% Formaldehyde for 30 mint and subsequently washed with PBS. Cell

membrane Fc receptors were blocked with 1 % BSA for 30 minutes at room temperature to prevent background staining. It is thought that background staining occur as a result of either non-specific antibody (Ab) binding to endogenous Fc receptors (FcRs) or a combination of ionic and hydrophobic interactions. After washing with PBS fixed HaCaT cells monolayer was incubated with Goat anti hBD2 at a (1: 250) ratio primary antibody overnight in 0.3 % BSA and kept it in the fridge. Cells were washed again with PBS and incubated with FITC- labeled appropriate secondary antibody at a (1:500) dilution then leave it for 2-3 hours in the dark at +4°C. After three hours the chamber wells were washed with PBS and then left them to soak in the PBS to avoid them to be dried up. Representative pictures were taken by confocal microscope.

2.2.11 HaCaT cell permeability measured by FITC-albumin assay

For detection of macromolecular passage across the transwell culture insert, a tracer solution containing FITC-alb was prepared in complete medium. HaCaT cell were grown on the transwell insert growth surface area of 0.33 cm^2 with 0.45 µM pore size transwell with cell density of 2 $x10^4$ cells/cm^2. Before starting the experiment standard curve was obtained for FITC-alb with the concentration of 1M, 500mM, 250mM at 480/20 excitation and 590/25 emission wavelength.

For the permeability assay, 0.02 % FITC-alb was added to the apical side of the transwell and sample were collected at several time points from the lower compartment of the transwell. For inhibition assay test wells were incubated with inhibiting peptides 2-3 hours before adding V8 protease with either 4µg/mL or 8µg/mL. The culture plate was

kept in the incubator for an hour. The plate was kept on the steady shaker for 15 mint at 37°C with steady speed of 300 HTZ to equalize FITC-alb on the top of the monolayer. Then 10 µL of the filtered media was drawn from the lower compartment of the transwell and transfer it to 96-well plate. For the detection of FITC-alb, 96-well plates were read on a plate reader at emission and excitation wavelength of 485 and 590 nm, respectively. In order to allow qualitative comparison, the data is presented and expressed as relative permeability (with respect to a control stimulus run in each individual experiment) intensity of the FITC-alb permeabilized media at 485/590 nm was compared with the intensity of empty well with no monolayer. This process was repeated after 8 and 24 hours where each time withdraw 10 µL of permeabilized media was drawn from the lower compartment of the transwell and transfer it to 96-well plate to measure increase in fluorescence intensity. HaCaT cell monolayer damage by V8 protease was viewed as a loss of cell- cell contact. The damage to the HaCaT cell monolayer was more prominent when size of the gaps between the cells was increased. The increase in the gap enables FITC-albumin to permeabilize through these gaps rapidly to enter into the bottom part of the well. Ultimately fluorescence intensity of the medium present in the bottom part of the well was increased.

2.2.12 Role of Vitamin D in protecting skin barrier by expressing hBD2

Trans-epithelial electrical resistance (TEER ohms/cm2) was measured by using Millipore protocol provided in the Ohms meter manual. HaCaT cells were grown on Transwell polycarbonate microporous filters with a pore size of 0.45 µM (corning costar UK). Control and Vitamin D3 treated HaCaT cells grown on transwells were exposed to V8 for

24 hours. TEER measurement was repeated every day and culture medium was changed daily. Each insert was measured at 3 different sites and mean values were calculated. A control without cells was also performed. All the experiment performed in duplicate with n=3. Damage to HaCaT cells monolayer by V8 protease was visualized under the light microscope.

HaCaT cells monolayer resistance was obtained by subtracting resistance reading across the blank insert from the resistance reading of the cell culture insert. The resistance is inversely proportional to the area of the monolayer, the larger the membrane lowers the resistance. After measuring the resistance, HaCaT cell monolayer treated with 1μM/mL 25 OH vitamin D3 and 1,25 OH vitamin D3 for 24 hours. After 24 hours serum + medium was replaced with serum free medium. Vitamin D3 treated and non-treated HaCaT cell exposed to 4μg/mL of V8 for another 24 hours. Pictures were taken before measured the TEER. Unit area resistance = resistance (Ω) x effective membrane area (cm2). The unit area resistance is obtained by multiplying the meter readings by the effective surface area of the filter membrane, where area is the surface area of the HaCaT cell monolayer. The resistance is inversely proportional to the surface area. Thus larger the membrane, the lower the resistance.

2.2.13 Quantitative Reverse Transcription Polymerase Chain Reaction (QRT-PCR)

Total RNA was extracted from HaCaT cells by (QIAGEN RNA extraction kit) according to manufacturer's instructions using reagent (RLT, RW1, RPE buffers, β-

mercaptoethanol, proteinase K solution, Nuclease free water, and Dnase 1 solution). Following this 100ng/µL of total RNA was then reverse transcribed using the cDNA synthesis kit (RNA at 100ng/µL, Taqman reverse transcription reagent kit, Nuclease free water). The mRNA level of DEFB4 (genetic name of hBD2) was examined using pre-developed Taqman assay (TaqMan Gene expression Assay consist of a pair of unlabled forward and reverse PCR primers and a TaqMan probe with a FAM dye label on the 5 end and minor groove binder (MGB) and non fluorescent quencher on the 3 end). (Master Mix 12.5 make 14x=175, 18s 1.25 14x=17.5). The QRT-PCR was performed to find about the mRNA expression level of hBD2 in human keratinocyte cell line HaCaT cells by comparing it with 18S ribosomal RNA as a control. 18S rRNA is a component of the small eukaryotic ribosomal subumit (40S) and structural RNA for the small component of ribosome's and thus one of the basic components of all eukaryotic cells. The genes coding for 18S rRNA are referred to as 18S rDNA. The 18S rRNA gene is one of the most frequently used genes in polygenetic studies and an important marker for random target PCR in environmental biodiversity screening.

2.2.14 ELISA to determine hBD2 in the CM

hBD2 concentration present in the CM was determined by ELISA assay. The protocol provided by (hBD2 ELISA development kit Peprotech USA) was followed. The standard hBD2 peptide provided in the ELISA development kit was serially diluted in diluent solution (PBS + TW-20 and BSA). The 96 well ELISA plate was coated with goat anti hBD2 Capture antibody provided in the ELISA development kit and keep the plate at room temperature for overnight. The coated Plate was washed 4 times with PBS+ 0.05 %

v/v TW-20 and then blocked with 1% BSA in PBS for 1hour at room temperature then wash it 4 times with PBS+TW-20. The standard peptide (recombinant hBD2 from the kit) was serially diluted (1000 pg/mL to zero) with diluents 0.1% BSA+0.05% TW-20 and then poured into the plate. Immediately added 50 µL of each standard and sample to each well in triplicate and incubate at room temperature for at least 2 hours. Aspirate the wells and after 4 washes with PBS+TW-20 added 50 µL of biotinylated detection antibody diluted at 0.5µg/mL and incubate at room temperature for 2 hours. After 4 washes the antibody was detected with Avidin-HRP conjugate 1:2000 ratio in dilution by incubating 30 minutest at room temperature. After 4 washes added 50 µL of ABTS Substrate in each well and incubate at room temperature for color development. Monitor the color development with plate reader at 405 nm with wavelength correction set at 650 nm.

2.2.15 Data presentation and statistical analysis,

2.2.15 A Measures the size of holes in the monolayer with ImageJ

Image J software was used to measur the size of the holes formed by inducing *S.aureus* proteases in the HaCaT cells monolayer's.

In the tool option of Image J software, 'Freehand was selected to draw an outline around the damaged area. Once the outline around all the damaged areas of the image was drawn, to get the measurement it need to click on 'Analyze > Measure. In the result section damaged area of the image is defined as the units- pixels found in that area. Then the total area of the image which is define as the the total pixel area of the image can be drawn by highlighting the whole image. Then from the 'Edit >Selection > command was

used to select the whole image. The command 'Analyze > Measure' was used to get area of the entire image. The Percentage of damaged area can be calculated by using following formula

= (Outline damaged area of the image/ Total area of the image) *100

The calculated damaged area of the monolayer can be define as 'percentage area damage which is showed in the image.

2.2.15 B Calculate W.B band intensity by using Image J software

The intensity of the band in the W.B analysis can be calculated by using Image J software, which is a semi-quantitative tool. The Rectangular tool was selected to highlight the band of interest. The gel was analyzed by using commands Analyze > Gel > Gel analyze option > uncheck the box for invert peaks but keep the default value and clik o.k. After getting peaks for all the bands then choose the command Analyze > Gel > Plot lanes which provides values to the selected peaks. In order to prevent the overlapping of the peaks to each other the segmented free straight line tool was selected. This command makes straight line and separates each peak from the other peak. By selecting Wand (tracing) tool and clicking the peak give values to each peak. Then go to Analyze > Gel > Label the peak, that will provide the percentage area. Get the percentage by dividing the highest value by itself and then the other values divided by the highest multiply by 100.

2.2.15 C easuring cell Fluorescence using image J software The fluorescence density of the monolayer can be detected by Image J software**.** In the Image J software from the 'Analyze menu' select "set measurement". Select Area, Integrated Density and

Mean gray value. Now select "Measure from the analyze menu. A box will pop up with a stack of values for that cell then select the region near the cell which has no fluorescence that will become the background value. It needs to take three selections from around the cell. Repeat these steps for the rest of the cells and figures.-Once it done then copy all the data into the new Excel worksheet. Following formula can be used to calculate the corrected total cell fluorescence (CTCF).

CTCF=Integrated Density-(Area of selected cell x Mean fluorescence of background readings)

2.2.15 D One way ANOVA (unstacked)

One-way analysis of variance technique was used to compare means of my numerical data from three or more samples in an experiment. Graph Pad Prism and Minitab 16 software were used for the statistical analysis. The ANOVA tests the null hypothesis that samples in two or more groups are drawn from populations with the same means value. Typically, however, the one way ANOVA is used to test for differences among at least three groups, since the two-group can be covered by a t-test. When there are only two means to compare, the t-test and the F-test are equivalent; the relation between ANOVA and t is given by F=t2. The relation of the values within the group was testified by using Tukey's multiple comparison posttest.

Chapter 3

3-Staphyloccocus.Aureus secreted proteases can damage HaCaT cell monolayer

3.1 Introduction

S.aureus infection is very common in atopic dermatitis patients, 90% of AD patients are *S. aureus* carriers, having the bacteria present in their nasal and perineal areas. Although *S. aureus* is not part of the normal skin microflora, it is considered an environmental factor in the pathogenesis of AD **(Collins et al., 2013).** *S. aureus* disease causing ability is due to expressing a variety of virulence factors, those are surface proteins, enzymes, toxins, and antigens. These virulence factors play a critical role such as, adherence to the surface, invasion of the host and avoidance of the immune system, which cause harmful toxic effects to the host. The major proteolytic enzymes secreted by *S. aureus* are aureolysin, V8 and two cysteine proteases - staphopain A and staphopain B **(Arvidson S et al., 2000).)**. *S.aureus* cysteine protease staphopain B is neutralized by staphostatin secreted by the bacteria. The staphopain B secreted as a zymogen, which is proteolytically processed by V8 protease, as outlined in chapter 1 **(Chan *et al.*, 1998; Rice *et al.*, 2001).** Staphopain B is encoded by the *sspB* gene, which is located in the operon contiguous to the gene for the serine protease sspA **(Chan *et al.*, 1998; Rice *et al.*, 2001).** *S. aureus* proteases are considered virulence factors

and by damaging epidermal permeability barrier cause exacerbation of AD, which increased sensitization to allergens **(Shaw *et al.*, 2004).** Specifically, one study, done in 2002 by Medzobrodzki *et al.*, states that, *S. aureus* strains isolated from the skin lesion of 26 AD patients, secrete proteases. This study describes the presence of proteolytic activity in the lesional skin of AD which provided a link how *S.aureus* proteases may contribute to the pathogenicity of AD. Like other extracellular proteases V8 is involved in *S.aureus* colonization and infection of human tissue **(Massimi et *al.*, 2002).** *S. aureus* V8 (sspA) a serine protease with molecular weight of 27 KDa at pH 4.0 - 7.8 specifically cleaves peptide bonds on the carbonyl side of either aspartic or glutamic acids (Endoproteinase-Glu-C) **(Prasad *et al.*, 2004)**. V8 is secreted in a pro-form, which is proteolytically cleaved to produce a mature and functional enzyme, possibly in an aureolysin-dependent manner **(See page76) (Drapeau *et al.*, 1978; Lindsay *et al.*, 1999)**. V8 is a most important protease for the degradation of fibronectin binding protein and surface protein "A" which are involved in adherence to host tissue **(Mcgavin *et al.*, 1997** & **Karlsson A et al., 2001)**. In reference to disease-causing toxins, *S. aureus* secreted exfoliative toxin (ET) is a virulence factor. The ET belongs to the family of disease-causing toxins that can damage the skin barrier by protease activity **(Amagai *et al.*, 2000)**. The Exfoliative toxins can degrade adhesional junction protein desmoglein-1 (DMG-1) in a dose dependent manner without any contributing factors. The separation of the epidermal layer at the stratum granulosum results either directly or indirectly from the enzymatic activity of exfoliative toxins (**Bailey CJ& Redpath MB, 1992)**. The proteolytic activity of ET starts when highly charged N-terminus bind to desmoglein-1 and causes a conformational change which exposes the active site. After binding to the active

site ET cleaves the protein between the third and fourth extracellular domain **(Dancer *et al.*, 1990).**

The sequence analysis of the ET revealed homology to V8 protease, having serine-histidine-aspartate catalytic triad which is present in all serine proteases **(Figure 3.1).** The mutation of any of these three amino acids results in loss of exfoliative activity in the newborn mouse model **(Gemmell *et al.*, 1997).** Exfoliative toxins (ET) homology to serine proteases proven by exhibiting esterase activity, an activity commonly associated with serine proteases **(Dancer *et al.*, 1990).** The supportive evidence that exfoliative toxins act as serine protease was provided when serine residue of the active site of ET-A was replaced with alanine. The mutated ETA toxin was able to bind, but unable to cleave desmoglein-1 **(Yamaguchi *et al.*, 2002).** This evidence explains how both ET and V8 have capacity to damage skin barrier by having serine protease activity. *S. aureus* virulence factors (as outlined in chapter 1) are regulated by an accessory gene regulator (Agr): a quorum-sensing system. Bacteria use quorum sensing to co-ordinate certain behaviours such as biofilm formation, virulence, and antibiotic resistance, based on the local density of the bacterial population **(Lerat E et al., 2004)**. The Agr operon consists of two divergently transcribed promoters, P2 and P3, which encode a quorum-sensing system and a pleiotropic regulatory RNA molecule, respectively **(Roux A et al., 2014).** Operon is a stretch of DNA found in bacteria, operon controls gene regulation having three components, which are promoter, operator, and structural gene. Structural sequence codes for protein, RNA polymerases binds with promotor sequence, repressors binds to the operator sequence and inhibits RNA polymerases from binding. A regulon is a group of genes all needed for the same process but physically located in different

parts of the chromosomes and have their own promoters **(Blumenthal et al., 2004)**. Auto-inducers are the signaling molecules produce and secrete by certain bacteria's, which also have receptors that specifically detect the inducer **(Novick *et al.*, 2008).** To study the role of *S. aureus* secreted proteases in the breakdown of skin barrier, *S. aureus* parent strain 8325-4 which is a standard laboratory strain and their laboratory generated mutants strains were kindly donated by (Dr Fiztgerald department of microbiology Royal (Dick) School of Veterinary Sciences Edinburgh). The mutants were constructed by the insertional inactivation technique as described previously **(Karlsson et al., 2001) (Shaw et al., 2004).** Insertional inactivation is a technique, where a plasmid is use to disable expression of a gene. Shaw described how mutant strains were constructed by inactivation of either V8, staphopain A or staphopain B secreted proteases. Selectively regulating gene expression in bacteria by adding 5mg/ml of erythromycin in the growth medium, has provided an important tool for studying gene function. **(Novick et al., 2000).**

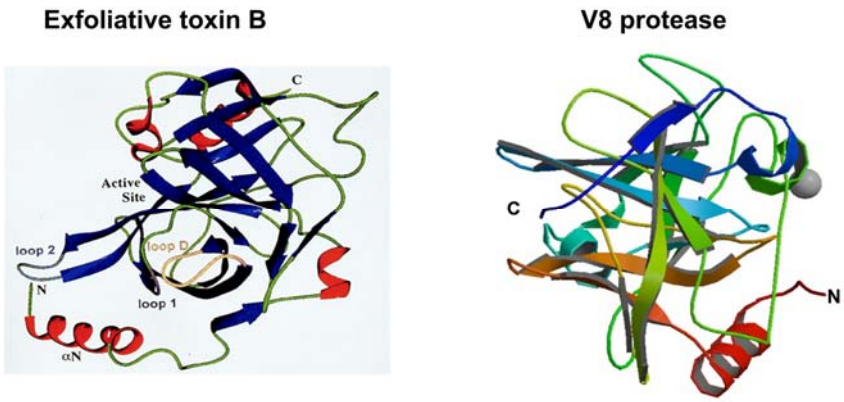


Figure 3.1 The structural homology of exfoliative toxin B to V8 protease

Schematic drawing shows *S.aureus* exfoliative toxin "A" having structural homology to V8. In the secondary structure of ETB, α-helices are shown in red, β strands are in blue; loops 1, 2 and' D' are in magenta, cyan, and yellow, respectively. The same color presentation is for V8 protease. (Picture is taken from (RCSB) protein data base with open access permission for 1QTF) **(Vath, G.M *et al*., 1999)**.

The selective modulation of gene expression levels is produced which allows the creation of conditional lethal phenotype, which is a powerful strategy in determining the contribution of phenotype when genes are essential. The following are the mutants which were used in the experiments; **L17** (with insertional inactivation of sspB (Staphopain B) of *S. arueus* strain 8325-4-SspB), **L22** (with insertional inactivation of sspA (V8) *S. arueus* strain 8325-4 sspA), **L27** (insertional inactivation of scpA (Staphopain A).

The virulence factors, present in the supernatant of the parent and mutant strains were applied to the HaCaT cell monolayer. The Proteolytic activity associated with recombinant V8 and serine proteases which were present in the supernatant was analyzed separately by using SDS page and zymographic analysis, (The zymographic method described by **Arvidson *et al.*, 1973** was followed) **(See page106).** The separation occurs in a polyacrylamide gel containing a specific substrate that is co-polymerized with acrylamide (**Fernandez-Resa et al., 1995).** *S. epidermidis is a skin* commensal and the role played by *S. epidermidis* for the protection of skin barrier against *S. aureus* proteases was also studied **(See page 104).**

Aim:

The structure of the study was designed in such a way to identify the most important virulent factor secreted by *S.aureus,* which played a significant role in breaking

the skin barrier. The deleterious effect of *S.aureus* secreted serine protease is compared with other proteases.

3.2 Results

S. aureus parent strain 8325-4, and its derivative RN6390, SH100, and the skin commensal S. *epidermidis*, were cultured to late stationary phase in 10 mL of Tryptic Soya Broth (TSB) without antibiotic. Mutant strains were similarly cultured in TSB broth, but in the presence of erythromycin. The concentration of bacteria in a suspension was measured by calculating OD (optical density) in a spectrophotometer **(See page 105).**

3.2.1- *S.aureus* secreted protease can damage HaCaT cell monolayer

Confluent monolayers of HaCaT cells grown in 1 mL of serum free medium (SFM) per well were exposed to 40 µL of supernatant obtained from *S.aureus* strains 8325-4, RN6390 and SH100. The 40 µL of supernatant in 1 mL of SFM, 0.004 dilution factor (DF) is considered as minimum effective concentration of supernatant, which was optimized after a series of experiments.

Differential interface microscopy (DIC microscopy) and with phalloidin-FITC staining revealed: Control normal HaCaT cell monolayers revealed no damage without any gaps between the cells **(Fig 3.2 a)**. Supernatant from RN 6390 caused retraction of the cell margins and monolayers displayed increased gaps between the cells after 12

hours **(Fig 3.2 b).** Control HaCaT cells exposed to 8325-4 revealed damage to the monolayer and displayed increased gaps between the cells after 12 hours **(Fig 3.2 c).** Damage to the HaCaT cell monolayers was observed with supernatant from SH100 after 12 hours **(Fig 3.2 d).** HaCaT cells exposed to the supernatant derived from mutant strain L17 revealed normal-looking monolayers without any damage after 12 hours **(Fig 3.2 e).** HaCaT cells exposed to the supernatant derived from mutant strain L22 revealed normal-looking monolayers without any damage **(Fig 3.2 f).** Percentage damage to the HaCaT cells by exposing supernatant of different strains compared to unexposed HaCaT cells for 12 hours which had 0% damage. Where I observed 20% damage in the monolayer exposed to RN6390, 22% with 8325-4, 15% with SH100 **(Fig 3.2 g)** which was quantified by using Image J software.

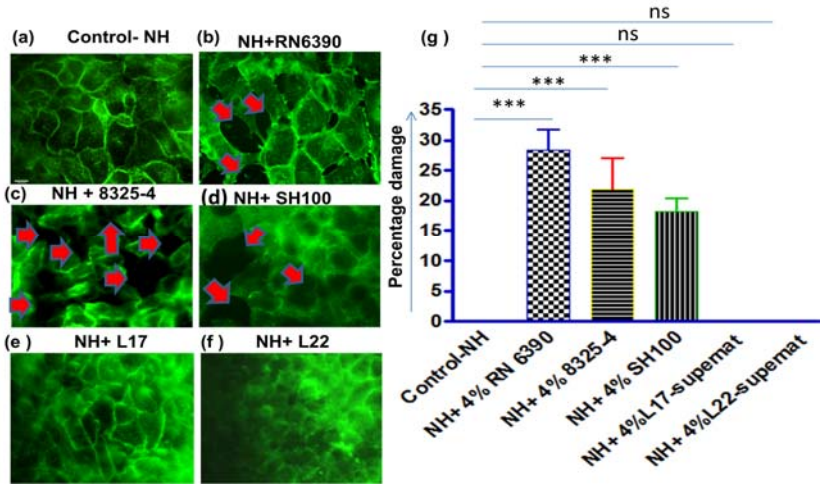


Figure-3.2 HaCaT cell monolayer exposed to *S.aureus* CM-DIC microscopy and with phalloidin-FITC staining revealed. (a) Control normal HaCaT cell monolayers (b) Control-Normal HaCaT cells exposed to 0.004 DF Supernatant of RN 6390. 12 hours (c) Control-Normal HaCaT cells exposed to 0.004 DF Supernatant of 8325-4. (d) Control-Normal HaCaT cells exposed to 0.004 DF Supernatant of SH100. (e) Control-Normal HaCaT cells exposed to 0.004 DF Supernatant of mutant strain L17. (f) Control-Normal HaCaT cells exposed to 0.004 DF Supernatant of mutant strain L22. Data is representative of n=4 experiments perform with replicates (one way ANOVA) Magnification x400. **Tukey's Multiple Comparison Test** (Control-NH vs NH+ 0.004 DF RN6390 $p<0.001$) (Control-NH vs NH+ 0.004 DF 8325-4 $P < 0.001$) (Control-NH vs NH+ 0.004 DF SH100 $P < 0.001$) (Control-NH vs NH+ 0.004 DF L17-supernat ns) (Control-NH vs NH+ 0.004 DF L22-supernat ns).

3.2.2 *S.aureus* exotoxins promote cell permeabilit

To assess the toxic effects of *S.aureus* CM on cell viability, we stained live cell culture with Trypan blue and noted that as early as 2 h, RN6390 CM caused widespread staining, even in cells that appeared viable and fully adherent by light microscopy **(Figure 3.3 a).** An increase in cell permeability, as assessed by Trypan blue **(t=2h, Figure 3.3 a)**, by reducing the dilution factor which are as follow 0.01 0.02, 0.05, 0.1, 0.2, which reveals that 0.05 dilution factor of 50 µL of added which reveals that *S.aureus* RN 6390 CM with dilution factor of 0.05 was sufficient to cause approx. 50% of all cells to stain positive (a consistent observation within a 10% margin of error when comparing different batches of RN6390 CM; data not presented). To block the activity of the proteases present in RN6390 CM Leupeptin is used. Leupeptin is a naturally occurring protease inhibitor that can inhibit cysteine, serine and threonine peptidases. Leupeptin used with concentration of 4 µg/mL. Leupeptin cause decrease in Trypan, Blue permeability by having Protease inhibitor activity which ultimately reduced LDH (Lactate dehydrogenase) secretion. Where levels are compared with the total amount of LDH released from lysed cells following exposure to 0.1% Tween20. LDH assays also confirmed our preliminary observation with Trypan blue that an incubation of at least 60 min was required before a significant increase in permeability was observed. The ability of RN6390 CM to cause approx. 50% permeabilization after 120 min was assayed for the release of cytoplasmic LDH **(Figure 3.3 b).** Thus, RN6390 CM caused a loss in plasma membrane integrity.

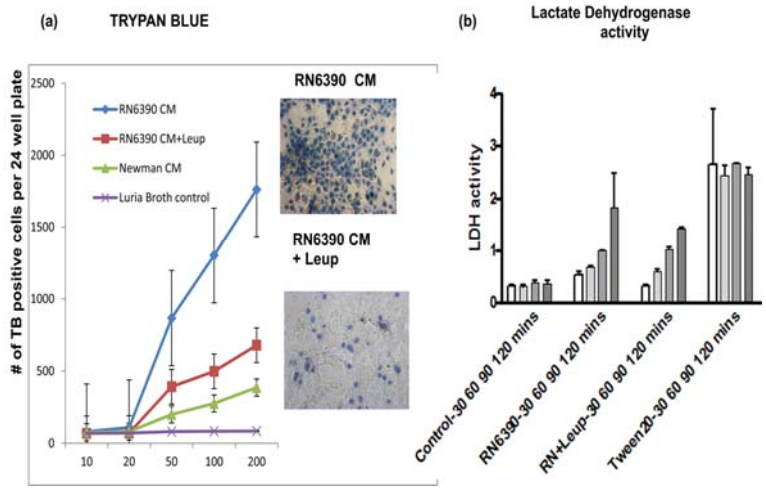


Figure-3.3: Quantitate the deleterious effects of RN6390 CM for HaCat viability. (a) HaCat cells exposed to LB media, RN6390 CM with increasing volume, which reveals that decreasing dilution factor of RN6390 CM was sufficient to cause approx. 50% of all cells to stain positive (b) RN6390 CM cause approx.50% permeabilization after 120 mint and LDH levels are compared with the total amount of LDH activity released by cells following exposure to 0.1% Tween20 serving as a positive control for 100% permeabilization. In addition to adding LB, some assays also contained Leupeptin to block the protease activity. n=3 experiments and always performed with replicates. Values are mean ± SE of three independent HaCat cultures with triplets. (Two way ANOVA) Magnification x200 Control =ns $P > 0.05$ RN6390 ** = $P<0.01$ RN+Leup * = $P < 0.05$ Tween20 = ns $P > 0.05$-

Bonferroni post –test Compare the changes in LDH release after 30 Mins vs 60 Mins, 30 Mins and 90 Mins, 30 Mins and 120 Mins-LDH release 30 Mins vs LDH release 60 Mins

Control P > 0.05 RN6390 P > 0.05 RN+ Leup P > 0.05 Tween 20 P > 0.05

LDH release 30 Mins vs. LDH release 90 Mins

Control P > 0.05 RN6390 P > 0.05 RN+Leup P > 0.05 Tween 20 P > 0.05

LDH release 30 Min vs. LDH release 120 Mins

Control P > 0.05 RN6390 P<0.01 RN+Leup P<0.01 Tween 20 P > 0.05

3.2.3 SDS-PAGE gel analysis for the proteases in the CM *S. aureus* RN6390 and *S. epidermidis* supernatant were loaded with an equal amount on to the SDS-16% polyacrylamide gel. Protein marker was applied to the gel in the separate lane to assess the molecular weight of the protein, present in the supernatant. Electrophoresis was performed. Protein bands were visualized after destaining the gel in Coomassie destains solution -2-3 hours. There were no bands found in the supernatant derived from *S. epidermidis,* whilst multiple bands with different sizes were visible in the *S. aureus* supernatant. A 17 kDa band, suggestive of staphopain B, and another prominent band at 27 kDa, which is the expected size of V8, was visible. An additional prominent, 37 kDa band, the expected size of Aureolysin, was found in the *S. aureus* supernatant **(Fig 3.4).** I could say from these results that proteases are present in the CM obtained from *S.aureus* RN6390 and not in the CM obtained from *S.Epidermidis.*

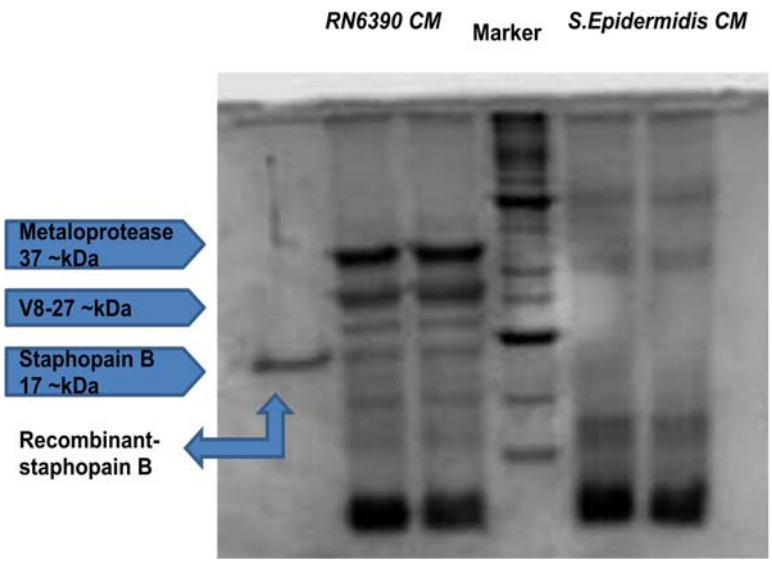


Figure 3.4 SDS-PAGE gel analyses for the proteases present in the CM.

To determine the presence of proteases in the supernatant and their activity, SDS page gel analysis was performed **(a)**. No bands were found in the supernatant derived from *S. epidermidis,* whilst three prominent bands were visible in the *S. aureus* RN6390 supernatant samples. The sizes of these bands were relevant to the standard marker.

3.2.4 Zymogram gel analysis for the proteases in the CM

Zymography is defining as simple, quantifiable and functional assay to analyze proteases in biological samples. In zymography, the proteins are separated by electrophoresis under non-reducing conditions. The separation occurs in a polyacrylamide gel containing a specific substrate that is co-polymerized with acrylamide (**Fernandez-Resa et al., 1995).**

Zymogram gel analysis was performed to determine proteolytic activity of the proteases present in the supernatant of 8325-4, SH100, RN6390. *And Mutant strains L27, L22, and L17.* The zones of hydrolysis of the substrate were visualized after staining with coomassie blue and then destaining gel in Coomassie destains solution. There was a clear, whitish area visible in the prospective lanes of samples from 8325-4; SH100 and RN6390. There was no visible destained area in the lanes of L17, L22, L27 and S. *epidermidis*. **(Figure 3.5).** The proteases are detected as clear bands against a blue background of undegraded 0.1 % β-casein present in the gel. The destained band was visible at 27kDa, in the corresponding lane of V8, 8325-4; SH100 and RN6390. In another experiments I loaded recombinant V8 proteases along with supernatant from 8325-4; SH100 and RN6390 on the 0.1 % β-casein zymogram gel.

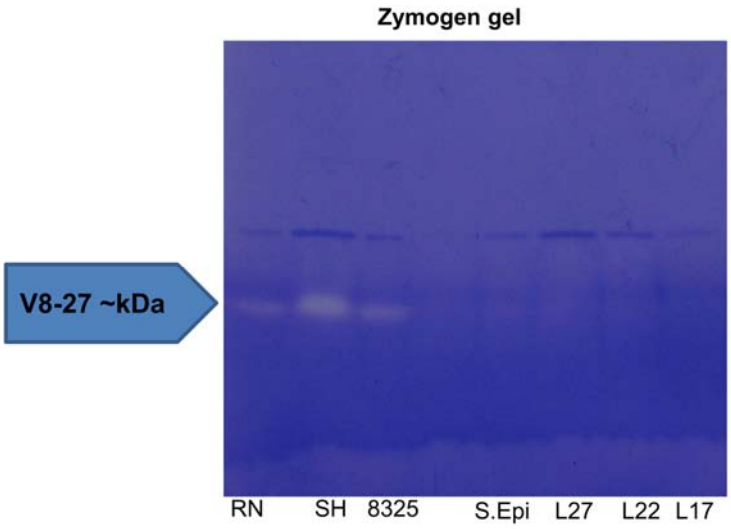


Figure-3.5 Zymogram gel analysis for the proteases present in the CM.

Proteolytic activity of the proteases presents in the supernatant derived from the wild-type and mutant strains were quantified by zymogram gel. Clear areas were visible only in the lanes of RN, SH, 8324 strains. There was no visible destained area in the lanes of L17, L22, L27 and S. *epidermidis*.

3.2.5 *S.aureus* V8 protease can cause damage to the monolayer

So far I had found that toxic factors are present in the supernatants of RN6390, 8325-4 and SH100 which could damage the HaCaT cell monolayers after 12 hours but mutant strains L17, L22 and L27 were unable to damage the HaCaT cell monolayer. After the zymograph analysis, it shows that proteases are present in an active form in the supernatant which could degraded the substrate that proteases would have cause damage to the HaCaT cell monolayer. In this experiment I used only recombinant V8 after optimising used with concentration of 4µg/mL to see the extent of damage to the HaCaT cell monolayer.

Light microscopic examination revealed, there was no visible change in the unexposed control HaCaT cell monolayers. HaCaT cells monolayers induced with V8 started shrinking from their periphery and adopting rounded shapes after 12 hours, suggesting toxicity, and loss of cell-cell interactions. To better define the boundaries of individual cells, they were stained for intracellular filamentous actin, using phalloidin-FITC to reveal the proper cell shape by staining cortical cytoskeleton.

Control cells displayed normal HaCaT cell monolayers, without any gaps between the cells **(Figure 3.6 a)**. Prominent morphological changes were observed in the V8- induced cells after 24 hours, where V8 protease had disrupted the monolayer, as evidenced by a retraction of cells from each other and starting holes formation in the monolayer **(Fig 3.6 b).** This data demonstrate that recombinant V8 protease can replicate the HaCaT membrane damaging effects of *S.aureus* supernatant.

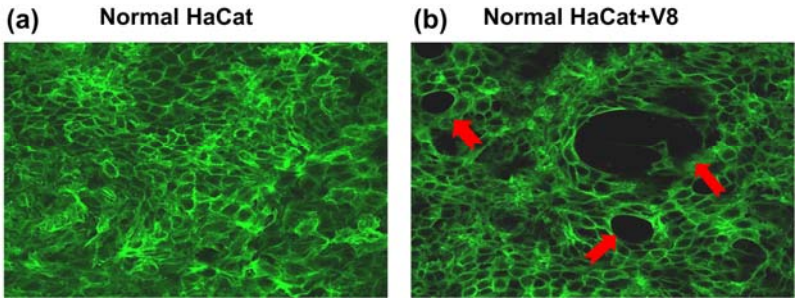


Figure-3.6 V8 protease can damage HaCaT cell monolayer

Confocal Laser microscopy and with phalloidin-FITC staining revealed: (a) Control normal HaCaT cell monolayers revealed cortical cytoskeleton and defined boundaries of individual cells, without any gaps between the cells after 24 hours (Fig a; Width in pixel 411, height in pixel 4, Font size 14). (b) Control HaCaT cells exposed to 4 µg/mL of V8 for 24 hours (Fig b; Width in pixel 413, hight 4, and Font size 14). Experiments were always performed with replicates: representative of n=4 at Magnification of x 200

3.2.6 Correlating damaging effect of Trypsin with V8 and staphopain B

To compare proteolytic damage to the HaCaT cells monolayer which could be caused by serine (V8) and (trypsin) or cysteine (staphopain B) proteases, a time course assay was conducted. After series of experiments I found that 0.004 dilution factor (DF) of supernatant effectively damage the monolayer. 4 µg/mL of V8 protease i.e 4 µg of neat protease in 1 mL of SFM effectively damage the monolayer. In the time-course study, HaCaT cell monolayers were exposed to 4 µg/mL of V8, trypsin or staphopain B, for 4 to 24 hours and assessed the extent of damage to the monolayer **(Figure 3.7).** There were no visible changes observed in the control unexposed HaCaT cell monolayers after 4, 8 and 24 hours **(Figure 3.7; lane 1st)**. The HaCaT cells monolayer exposed with 4 µg/mL of V8 show no changes after 4 and 8 hours. Whereas prominent morphological changes were observed (Yellow arrows) after 24 hours, which were loss in cell-cell contact and rounded hole formation. The image J quantification shows there was 22.16 % damage to the monolayer with V8 protease when compare to control which has no damage. **(Figure 3.7; lane 2nd)**. The morphological changes started in HaCaT cells after 8 hours when exposed to trypsin. These changes included loss of cell-cell contact, voids and fissure formation (Yellow arrows) pointed those changes. The image J quantification shows there was 17.15 % damage to monolayer compare to control which had 0% damage. There was prominent damage to the monolayer induced by trypsin after 24 hours (Yellow arrows). The image J quantification provided 41.36% damage to monolayer compare to control. **(Figure 3.7; lane 3rd)**. There were no such changes observed in the staphopain B-induced monolayers at 4, 8 and 24 hours timepoints **(Figure 3.7; Lane**

4th). This experiment shows that damage to HaCaT cell monolayer faster with trypsin compare to V8 protease (serine protease) while staphopain B (cysteine protease) was unable to damage HaCaT cell monolayer.

Lane 1st Lane 2nd Lane 3rd Lane 4th

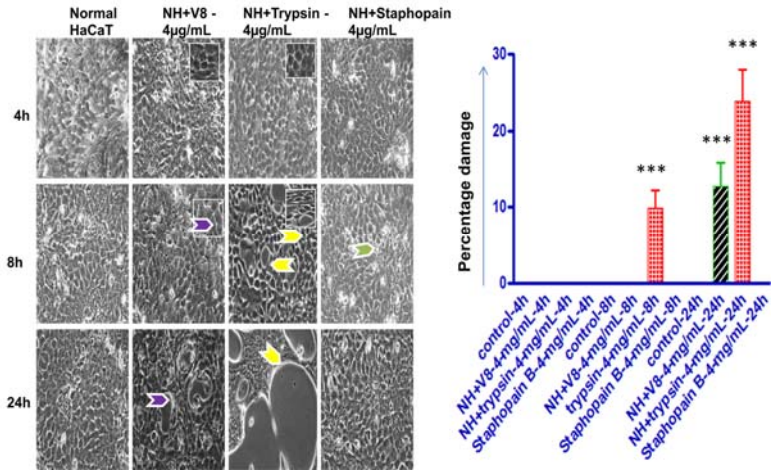


Figure-3.7 Optimizing monolayers damage with V8, trypsin and staphopain B. The time course, comparative study where HaCaT cells monolayers were exposed to 4µg/mL of V8, Trypsin and staphopain B for up to 24 hours. Lane 1st Normal HaCaT (unexposed) cell monolayers 4- 24 hours. Lane 2nd, Normal HaCaT cell monolayers exposed to 4µg/mL of V8 4- 24 hours. Lane 3rd Normal HaCaT cell monolayers exposed to 4µg/mL of Trypsin 4- 24 hours. Lane 4th, Normal HaCaT cell monolayers exposed to 4 µg/mL of staphopain B. (One way ANOVA n=3 magnification X200)

B. Tukey's Multiple Comparison Test -After 4h-(Control-4h vs NH+V8-4-mg/mL-4h $P > 0.05$) (Control-4h vs NH+trypsin-4-mg/mL-4h $P > 0.05$) (Control-4h vs Staphopain B-4-mg/mL-4h $P > 0.05$). After 8 h- (Control-8h vs NH+V8-4-mg/mL-8h $P > 0.05$) (Control-8h vs trypsin-4-mg/mL-8h $P < 0.01$) (Control-8h vs Staphopain B-4-mg/mL-8h

P > 0.05). After 24 h (Control-24h vs NH+V8-4-mg/mL-24h P < 0.001) (Control-24h vs NH+trypsin-4-mg/mL-24h P < 0.001) (Control-24h vs Staphopain B-4-mg/mL-24h P > 0.05)

3.2.7 V8 at higher concentrations can cause more damage

In this experiment higher concentrations of both V8 and staphopain B protease are tested on the HaCaT cell monolayer. The HaCaT cell monolayer was exposed to V8 and staphopain B, with concentrations of 4 and 8 µg/mL, for 24 hours. There was no change in the HaCaT cell monolayer after 24 hours in control unexposed HaCaT cell monolayers **(Fig 3.8 a).** Whilst there was loss of cell-cell contact, increase gap between the cells and observed holes formation in 4µg/mL of V8-treated monolayer after 24 hours (Image J quantification calculated as a % age damage provided 24.37% damage to monolayer compare to control **(Fig 3.8- b)**. These changes were more extensive in the monolayer which exposed to 8µg/mL of V8. There was starting of holes formation after 12 hours in the monolayer exposed to 8µg/mL of V8. Whilst size of the holes was significantly increased after 24 hours. The percentage damage with 8µg/mL of V8 calculated with image J showed 43% damage to the monolayer compare to control. **(Fig 3.8 c)**. HaCaT cell monolayers exposed to staphopain B 4µg/mL and 8µg/mL did not show any changes **(Fig 3.8 d, e)**. Image J software calculation reveal 25 % damage to the HaCaT cell monolayer with 4µg/mL of V8 and 43% damage to the HaCaT cell monolayer with 8µg/mL of V8 after 24 hours **(Fig 3.8 f).** The time-course study demonstrated that 4 µg/mL of V8 could damage the HaCaT cell monolayer after 24 hours, but staphopain B at the same concentration did not have any effect.

Figure-3.8 The extent of damage increase by increasing V8 concentration

To see how much damage to the HaCaT cell monolayer could be caused by higher concentration of V8 and staphopain B. Light microscope examination revealed that. (a) Control (Normal HaCaT), unexposed HaCaT cell monolayer. (b) Normal HaCaT cell monolayer exposed to 4µg/mL of V8 for 24 hours**.** (c) Normal HaCaT cell monolayer exposed to 8µg/mL of V8 24 hours. (d) Normal HaCaT cell monolayer exposed to 4 µg/mL of staphopain B. (e) Normal HaCaT cell monolayer exposed to 8µg/mL of staphopain B (f) Percentage damage to the HaCaT cell monolayer was calculated by using image J software. (1 way ANOVA n=3 magnification X400)

Tukey's Multiple Comparison Test; (NH- NH+ V8 4µg/mL $P < 0.01$) **(**(NH- NH+ V8 8µg/mL $P < 0.01$) (NH- NH+ Staphopain B 4µg/mL ns) (NH- NH+ Staphopain B 8µg/mL P > ns)

3.3 Discussion

S. aureus is the leading cause of gram-positive bacterial infection and can play a major role in breaking the antimicrobial barrier in atopic dermatitis **(Aly *et al.*, 1977).** *Staphylococcus* commensal and pathogenic species colonize the skin, where both expresses proteases. The proteases, secreted by commensal contributes collaborative bacterial coexistence on the skin, while proteases from pathogenic bacteria are used as virulence factors, helping them to colonize skin. The pathogenic bacteria from these initial sites of colonization can disseminate into deeper layers of skin, possibly leading to the spread of infection.

Additionally *S. epidermidis*, a skin commensal, was used as a control. *S. epidermidis* a common bacterial colonizer of mammalian skin, also produce, cysteine and serine exoproteases with relatively low substrate specificity **(Dubin et al., 2001).** The commensal lifestyle of *S. epidermidis* is partly achieved by the expression of extracellular neutral metalloprotease (Sepa) **(Lai Yet al., 2007).** *S. epidermidis* plays probiotic function by preventing colonization of more pathogenic bacteria such as *S.aureus.*

Medzobrodzki *et al.*, states that, *S. aureus* strains isolated from the skin lesion of 26 AD patients, secretes proteases. *S. aureus* disease causing ability is by expressing a variety of virulence factors, which cause harmful toxic effects to the host. *S. aureus* is known to produce prominent extracellular proteases which includes serine, cysteine and Aureolysin **(Dubin *et al.*, 2002).** Like other extracellular proteases V8 is involved in *S.aureus* colonization and infection of human tissue **(Massimi et *al.*, 2002**).

We established a culture model of the HaCaT cell line to better understand the mechanism *by which S. aureus* secreted proteases can compromise the integrity of a skin cell monolayer. *S. aureus* strains commonly found in the lesional skin of atopic dermatitis patients were used in this experiment (see page 103).

Supernatant from *S.aureus* residential strains caused damage to the HaCaT cell monolayer, but supernatant from *S. epidermidis* was unable to damage the monolayer. Zymogen gel analysis demonstrated no proteolytic activity in the supernatant derived from the mutant strains. Additionally, supernatant from mutant strains had no obvious deleterious effects on the integrity of the HaCaT cell monolayer. Whilst 8325-4 and RN6390, SH100 supernatants had a profound effect and cause damage to HaCaT cell monolayer. These changes were more prominent when we staind HaCaT cells cytoskeleton with phalloidin-FITC.

The deleterious effect of serine and cysteine proteases were analyzed when the monolayer was exposed to recombinant V8 and staphopain B; however staphopain B was unable to damage the HaCaT cell monolayer even at higher concentration. These data suggest that V8 is the most active protease secreted by *S. aureus* into the supernatant and present in the conditioned media used in these studies. It would say, V8 could be the master protease secreted by *S. aureus* involved in deterioration of the skin barrier in individuals with AD. This observation is consistent with another study, demonstrating that extracellular proteases secreted by *S. aureus* cause epidermal barrier dysfunction **(Hirasawa *et al.*, 2009).** In that study, they applied V8 to the back of nude mice for one week, which induced permeability barrier dysfunction, evaluated by TEWL

(Transepidermal water loss). It is not just the bacterial proteases capable of breaking barrier, common allergens such as house dust mite's secreted serine and cysteine proteases can damage skin barrier **(Chapman et al., 2007).**

S.aureus may play a role in the chronicity and severity of AD through its release of super-antigenic exotoxin **(Leung et al., 1993).** In addition to their immunological effects these toxins may also directly damage the skin barrier. *S.aureus* produces proteases that break down corneodesmosomes by a mechanism similar to that of KLK-(Kallikrein-related epidermal serine proteases) peptidases **(Miedzobrodzki et al., 2002).** In addition *S.aureus* secreted sphingosine deacylase and glyceropospholipids that may interfere with the formation of the lipid lamellae **(Otto M et al., 2004)**

3.4- Conclusion

S. aureus proteases are environmental factors in the pathogenesis of AD and previous studies had demonstrated that extracellular proteases secreted by *S. aureus* cause epidermal barrier dysfunction. I tried to find out which protease has the most deleterious effect on the HaCaT cell monolayer. After this series of experiments, I concluded that *S.aureus* strains 8325-4, RN6390 secrete serine (V8), cysteine (staphopain B) and Aureolysine (Zn metalloprotease) proteases which are present in the supernatant. I found that V8 (*S.aureus* serine protease) is the most active protease when I compared it with staphopain B (*S.aureus* cysteine protease) recombinant proteases by applying them on HaCaT cell monolayer. I also compare V8 protease with trypsin which is human serine protease and found trypsin had more rapid effect that V8 protease (see page136). Therefore, the next aim of this study will be to find out HaCaT cell monolayer peptide which become substrate for this protease and endogenous inhibitors, which could block their proteolytic. Currently there is no topical protease inhibitor available which block the proteolytic effect of V8 protease. The aim of this study was to protect epidermal barrier of AD from *S.aureus* protease by having protease inhibitor action, which ultimately reduces the pathogenesis of AD.

4-A secreted factor produced by stimulated HaCaT cells can inhibit *S.aureus* secreted proteases.

4.1 Introduction

S. aureus colonization is very common in individuals with AD and several studies confirmed isolation of multiple *S. aureus* strains from lesional skin of patients with AD **(Miedzobrodzki *et al.*, 2002).** In individuals with AD, the epidermal permeability barrier could be damaged by *S. aureus* secreted proteases, which cause primary sensitization to allergens **(Shaw.L *et al.*, 2004).** In addition *S.aureus* serine proteases, protects bacteria against host defence mechanism, by cleaving heavy chains of all immunoglobulin classes, plasma protease inhibitor, and elastin. **(Jusko, M., et al. 2014, prokesova L et al., 1992)**. Perhaps V8 is the most abundant of *S.aureus* secreted proteases, which is also important for transition from adhesive to non- adhesive phenotype via degradation of fibronectin-binding proteins, and other proteins on the *S.aureus* cell surface **(Karlsson A et al., 2001).**

In order to find all about activation and secretion of endogenous inhibitors, the mechanism of activation pathways needs to be studied. The toll like receptors (TLRs) are primary, key sensors of invading pathogens, which recognize conserved pathogen-associated molecular pattern (PAMPs) of bacteria, viruses, fungi and protozoa and induce host antimicrobial defense (see page **Error! Bookmark not defined.**) **(Medzhitov *et al.*, 1997). T**he epidermal keratinocytes express TLR2 and TLR4 which are involved in pathogen-induced cellular responses. The pro-inflammatory cytokine IL-1 β form an

important part of the inflammatory response of the body against infection. IL-1β is produced by tissue macrophages, monocytes, fibroblast, and dendritic cells, B Lymphocytes, NK cells and epithelial cells keratinocytes. The recognition of pathogens by TLR in keratinocytes leads to the production of the pro-inflammatory cytokine **(Pivarcsi, A et al., 2003).** Keratinocyte TLR2 and TLR4 may play important role in the protection of the skin barrier against pathogens.

Part of the skin defense against *S. aureus* exoproteases can be maintained by *S. epidermidis* by releasing "Lipid S" a part of the cell wall LTA, into the medium during growth **(Schroder, N.W *et al.*, 2003).** *S. epidermidis* Lipid S is a short chain length form of LTA, which is a prominent exocellular antigen (**Elliott, T.S.J et al., 2000).** *S. epidermidis* Lipid S plays an important role during host bacterial infection by stimulating immune cells through TLR2, leading to the production of IL1-β, contributing to host defense against invading microbes **(Lambert, P. A et al., 2000) (Morath *et al.*, 2001).** In vitro studies demonstrated that cell wall component of *S.epidermidis*, are recognized by TLR2. It was observed that killing of bacteria has significant qualitative and quantitative effects on key aspects of innate responses in vitro. Heat killed *S. epidermidis* (HKSE) can stimulate TLR2 and induce the production of NF-kB and IL1β. Heat killed preparation engaged the NF-kB pathway but had significantly lower capacity to activate other immune pathways **(Strunk T et al., 2011).**

S.aureus purified cell wall bound LTA plays an important role during host bacterial infection by stimulating immune cells through TLR2, leading to production of IL1-β, contributing to host defense against invading microbes 50 **(Morath *et al.*, 2001).**

Morath had shown that commercial LTA is of poor quality and purity and may not be biologically relevant **(Morath et al., 2002).** After exposure to *S.aureus* or LTA (purified), keratinocytes release IL1-β in the culture medium within 1 hour, which is the pre-formed IL1-β that is stored in the keratinocytes, and then newly synthesized protein is secreted within a time frame of 3 to 24 hours **(Olaru *et al.*, 2010).**

Increased susceptibility to infections and *S. aureus* colonization in AD could be related to the dysfunction of host genes encoding proteins that regulate innate immune-responses, including TLR **(Howell *et al,* 2006).** TLR2-mediated IL1-β and TNF-α production by CD14 monocytes was found to be selectively impaired in patients with AD. The most remarkable reduction in TLR2-mediated proinflammatory cytokine production was observed in CD14 monocytes, which are functionally defective in their capacity to produce proinflammtory cytokines on TLR2 stimuli **(Hasannejad, H et al., 2007).**

The immune cells stimulation through TLR2 can be described, when a stable cells lines expressing TLR2 R753Q were constructed by transfecting with human PCDNA3.1- or TLR2-R753Q expression plasmid. In these cell lines, TLR2 coding region confirmed the changes from CGG to CAG at nucleotide position 2257. The TLR2-R753Q transfected cells translating an amino acid substitution from arginine to glutamine at position 753 (R753Q) having a single nucleotide polymorphism (SNP). More than 10% of patients with AD are heterozygous for TLR2-R753Q having a single nucleotide polymorphism (SNP). The TLR2 mutation modifies Toll-like receptor expression and cytokine production which may crucially modulate the pathogenesis of atopic dermatitis

(Mrabet-Dhabi *et al.*, 2008). The monocytes from AD patients with TLR-2 R753Q mutation produced significantly more IL-6 and IL-12 compared with that of AD patients with non-mutated TLR-2 upon stimulation with TLR-2 agonists (**Niebuhr M et al., 2008)**. The importance of TLR2 in controlling infections was observed in murine models where TLR2-deficient mice have a significantly higher mortality rate than wild-type mice, after intravenous administration of *S. aureus* **(Osamu.Takeuchi *et al.*, 2000).** Atopic dermatitis patients having TLR2 mutations, and such as pathway may be needed to express antimicrobial factors, which could protect skin barrier.

Vitamin D is a well-known factor which plays a key role in the maintenance of skin barrier function in individuals with AD. Vitamin D3 is also essential for normal keratinocyte development and function **(Bikll DD, *et al,* 2008).** The role of Vitamin D in inflammation was determined by Wang, when he described a strong molecular link between Vitamin D deficiency and the genetics of chronic inflammatory disease **(Wang.T.T *et al.*, 2004).** The vitamin D nuclear receptor (VDR) significantly affects 229 human genes many of these genes have long been associated with autoimmune diseases, cancers and also leads to the synthesis of antimicrobial peptide. A general appreciation for how 25-D and 1, 25-D competes for nuclear receptors gets to their opposing roles in the body. Under most circumstances, active form 1, 25- D act as the "on" switch and the inactive form, 25-D is the "off" switch. 25-D is not completely inactive, but it does not and cannot activate the VDR **(Marshall, T. G et al., 2008).** Exposure to injury and infection enhances production of 1, 25-D, which in turn leads to the expression of anti-microbial peptide (AMP) and activation of TLR2 **(Leow, L., et al.**

2011). The VDR activation by 1, 25 D, transcribes gene for the enzyme CYP24A1, which increases conversion of 1, 25-D into inactive metabolite. An activated VDR also controls 1, 25-D concentration by limiting transcription of the gene CYP27B1, which converts 25-D into 1, 25-D **(Kato, S., and et al. 2007).** The involvement of additional signalling pathways in the induction of AMP gene expression is especially important for the induction of the DEFB4 gene (hBD2). The induction of DEFB4 gene in macrophages requires TLR activation and the convergence of the IL-1β and vitamin D pathways (**Adams, JS et al., 2009).** In this chapter I will study, Vitamin D3 can protect the skin barrier against *S. aureus* secreted proteases, in fact a complete description of Vitamin D was presented in chapter 1. The epidermal permeability barrier depends upon the transcellular and Paracellular pathways. The transcellular pathway leads across the apical and basolateral cell membrane, whereas the paracellular pathway is directed through the tight-junction **(Tsukita S et al., 2001)**.

TJ's are the main sealing site of the paracellular pathway in epithelia composes of four-transmembrane proteins (claudin, occluding, tricellulin and JAM). The TJ proteins along with barriers formation makes paracellular channels, which are in concert with membrane channels, regulate transport of ions both in health and disease state. Additional attention was drawn to the ion permeability of the paracellular pathway, as pathology of inflammatory or infectious diseases turned out to be caused by paracellular barrier defect **(Mankertz, J., and J. D. Schulzke. 2007).** The role played by vitamin D in the maintenance of skin barrier function can be detected by measuring the transepithelial electrical resistance (TEER) of the monolayer. To assess, directly, the link between hBD2

expressions by vitamin D and permeability barrier function, I restored permeability barrier homeostasis artificially, by growing HaCaT cells on a transwell membrane.

Thus it is desirable to determine para- and transcellulr resistance separately. This cannot be achieved by conventional transepithelial resistance measurement. The fence function of the TJ is evaluated in monolayers cultured in transwell filters, by inserting a fluorescent label protein into the apical membrane and detecting if any fluorescent label reaches the bsolateral membrane .That's why we presented FITC-alb approach which could optimize between these two pathways. The experiment design used most frequently in cell culture study of TJ protein function is either an overexpression or a knock-down of the TJ protein of interest. A study done by King where Claudin-1 knock-down increases TJ permeability to FITC dextran's that has molecular weight in the range of 4 and 40 kDa which is a typical size range for allergens **(King TP et al., 1994).** The SDS page gel and zymogen gel analysis of the supernatant collected from three strains of *S.aureus* (8325-4, RN6390, SH100) showed a peptide with molecular weight equal to V8 was able to damage the HaCaT cell monolayer.

Aims:

The epidermal permeability barrier can be maintained by the proper functioning of epidermal para- and transcellular pathways. The barrier damaged in AD caused by *S.aureus* protease interpreted as damage to the TJ (tight junction) proteins. After analyzing the role of *S. aureus* proteases as environmental factors in the pathogenesis of AD, now it is important to find their endogenous inhibitors to elucidate the pathogenesis of AD.

4.2 **Results**

To test that how **"***S. epidermidis"* a skin commensal maintained and protects the skin barrier, the suspension contained *S.epidermidis* was heated at 100°C for 30 minutes. It was described before; that *S.epidermidis* when heated at 100°C could release a cell wall component (Lipid S) into the liquid medium. To investigate the protective role played by *S. epidermidis* secreted lipid S, HaCaT cells monolayer was stimulated with 100 µL of heat killed *S. epidermidis* (HKSE) suspension solution for twenty four hours **(See page 97).** After twenty four hours HaCaT cells stimulated with HKSE suspenation solution were then exposed to supernatant of *S.aureus* strain 8325-4 for 12 hours. **Yasuko** had said that HaCaT cells can act as immune cells when stimulated with inducers. The increased production of proinflammatory mediators IL-10, TNF-α, and nitric oxide (NO) induced by UVB is mediated via the mitogen activated protein kinase (MAPK) signalling pathway in human keratinocyte (HaCaT) cells (**Yasuko Mutou et al., 2010)**. Morath had shown that commercially available *S.aureus* LTA is of poor quality and purity and may not be biologically relevant **(Morath et al., 2002).** I proposed that addition of pro-inflammatory cytokine with *S.aureus* LTA, which may support the inducing action of LTA. Therefore I stimulated HaCaT cell monolayer with LTA in the presence of recombinant IL1β for 24 hours. HaCaT cell monolayers stimulated with IL1β/LTA were evaluated against proteases present in the supernatant of *S.aureus* 8325-4.

4.2.1 - HKSE stimulated HaCaT cells are protected from *S. aureus* protease.

Normal HaCaT cells unstimulated and HaCaT cell monolayers stimulated with HKSE for 24 hours in SFM were exposed to supernatant of strain 8325-4 for 12 hours. It was observed that morphological changes started after 4 hours in HaCaT cells when exposed to supernatant of 8325-4. The process was on-going and voids and fissures appeared after 12 hours **(Figure 4.1 b).** Damage to the monolayer was so extensive that the whole monolayer was destroyed after 24 hours and remnants were floating in the media. In contrast, HaCaT cell monolayers first stimulated with HKSE were subsequently protected from the deleterious effect of the proteases present in the supernatant of 8325-4 after 12 hours **(Figure 4.1 c).**

Percentage damage calculated by using Image J software shows where unstimulated, unexposed HaCaT cells had no damage. Unstimulated HaCaT cells monolayer exposed with 8324-5 supernatant had 30% more damage to the HaCaT cells monolayer compare with unstimulated, unexposed monolayer. Whilst HaCaT cells stimulated with HKSE CM were 95 % protected compare with unstimulated, unexposed HaCaT cells and had only 5% damage.

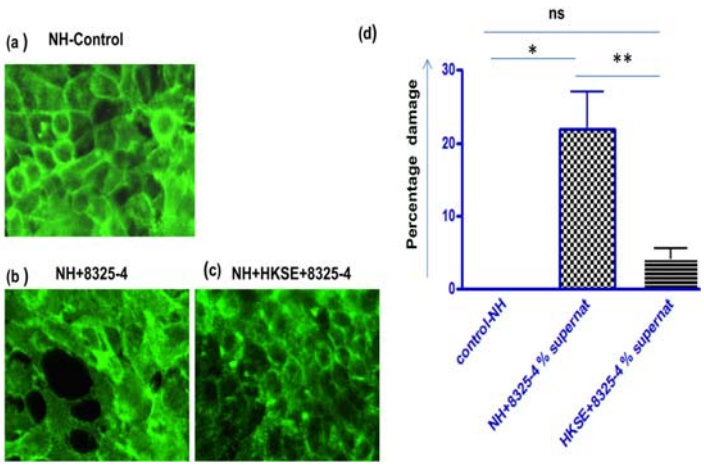


Figure- 4.1 HKSE stimulated HaCaT monolayer exposed to *S. aureus* CM.

DIC microscopy and with phalloidin-FITC staining revealed: (a) unstimulated HaCaT cells monolayer without exposure to proteases (b) Unstimulated HaCaT cell monolayers exposed to 0.004 DF supernatant of *S. aureus* wild type strains 8325-4 for 12 hours. (c) HKSE (heat killed *S.Epidermidis*)-stimulated HaCaT cell monolayers exposed to 0.004 DF supernatant of *S. aureus* strains 8325-4 for 12 hours (d) percentage damage calculated by using Image J software. Data are representative of n=4 experiments and performed with replicates. (1 way ANOVA) Magnification (100 X).

Tukey's Multiple Comparison Test

(Control-NH vs NH+ 0.004 DF supernatant of 8325- p **< 0.01) (Control-NH vs NH+HKSE- 0.004 DF supernatant ns) (NH+ 0.004 DF supernatant of 8325 vs HKSE+ 0.004 DF supernatant of 8325 P * < 0.05)

4.2.2 IL1-β/LTA stimulated cells are protected from the *S. aureus* proteases.

I proposed that addition of recombinant IL1- β may support LTA in the protection against *S. aureus*-secreted proteases. Recombinant IL1-β-100 ng + LTA 5 µg (active) in 1mL of serum plus medium was used to achieve protection against *S. aureus*-secreted proteases. HaCaT cells were stimulated with IL1-β/LTA for 24 hours, discard the old medium and after gentle wash with PBS, added fresh SFM. Unstimulated and IL1-β/LTA stimulated HaCaT cell monolayers were exposed to supernatant of *S.aureus* wild type strain 8325-4 for 12 hours.

Control normal HhaCat cells **(Fig 4.2 a).** Unstimulated HaCaT cells were unable to protect themselves against 4% supernatant of 8325-4, when exposed for 12 hours **(Fig 4.2 b).** HaCaT cell monolayers stimulated with IL1-β/LTA were protected from the damaging effect of the proteases present in the 4% supernatant of 8325-4 **(Fig 4.2 c).** ImageJ statistical analysis shows Unstimulated HaCaT cells had 25 % damage caused by 4% supernatant of 8325-4. Whilst IL1-β/LTA-stimulated HaCaT cell monolayers were protected and had only 4 % damaging effect of proteases from *S. aureus* strains 8325-4. Data are representative of n=4 experiments and performed with replicates.

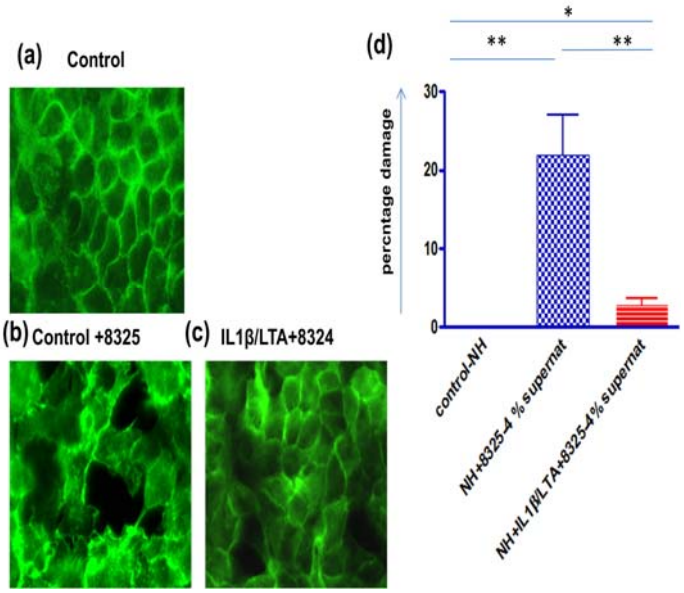


Figure 4.2 IL1-β /LTA stimulated HaCaT cells exposed to *S. aureus* CM

DIC microscopy and phalloidin-FITC staining revealed: (a) unstimulated HaCaT cells without exposure to supernatant. (b) HaCaT cell monolayers, without IL1-β/LTA, exposed to 4% supernatant derived from *S. aureus* wild-type strains 8325-4 for 12 hours (c) 24 hours IL1-β/LTA-stimulated HaCaT cell monolayers exposed to 4% supernatant of 8325-4 for 12 hours. (d) ImageJ statistical analysis Data is representative of n=4 experiments and performed with replicates. (1 way ANOVA).

Tukey's Multiple Comparison Test (Control-NH vs NH+8325-4 % supernat P ** < 0.01) (Control-NH vs IL1-β/LTA +8325-4 % supernat P * < 0.05) (NH+8325-4 % supernat vs NH+IL1b/LTA+8325-4% supernat P ** < 0.01)

4.2.3 Vitamin D3 can protect the skin barrier against *S. aureus* proteases

Having shown that exposure to LTA and IL1β could protect the HaCaT cells monolayer against damage induced by *S. aureus* secreted virulence factors. I then aimed to establish whether the presence of Vitamin D3 (which is protective in atopic dermatitis) could also have a protective effect for HaCaT cells from *S. aureus* secreted proteases. In these studies protection was assessed both by visualization of monolayer damage caused by proteases and by measurement of TEER (Transepithelial electrical resistance) which is a very well known measure of tight junction (TJ) function. In other words, TEER of the monolayer is inversely proportion to the TJ permeability. Hence, as TJ becomes less permeable, the TEER is increased. Conversely, as permeability goes up, TEER is reduced. The use of HaCaT cells offers the possibility of measuring TJ function in response to *S. aureus* proteases in a simple assay. Therefore, to measure the effect of *S. aureus* proteases on TJ function, HaCaT cells were cultured on a transwell membrane.

HaCaT cells were grown on transwells with a pore size of 0.45µm **(See page 112).** Once HaCaT cells became confluent, the TEER was measured, both in unstimulated and stimulated monolayers, before the start of the experiment. Light microscopic examination revealed voids and holes formation in the unstimulated monolayer when exposed to 4µg/mL of V8 for 24 hour. When I measured the TEER, there was significant drop in the TEER reading in unstimulated monolayer when exposed to 4µg/mL of V8 for 24 hour **(Fig 4.3 b).** In contrast, 25 OH Vitamin D3 -pre-stimulated HaCaT monolayers, TEER was increased and there was no damage to the monolayer after 24 hours of exposure with V8 protease **(Fig 4.3 c).** TEER was increased in the

monolayer treated with 1, 25 OH Vitamin D3 and there was no damage to the monolayer after exposing with 4µg/mL of V8 for 24 hours **(Fig 4.3 d).** Control unstimulated and unexposed normal HaCaT cell monolayer had resistance of 100 ± 30 ohms cm2. Whereas HaCaT cell monolayer exposed to 4µg/mL of V8 resistance was dropped up to 50 ± 30 ohms cm2. HaCaT cell monolayer stimulated with 25 OH Vitamin D3 then exposed to 4µg/mL of V8 had resistance of 150 ± 30 ohms cm2, which was slightly more than the control. HaCaT cell monolayer stimulated with 1, 25 OH Vitamin D3 then exposed to 4µg/mL of V8, there was gain in the resistance and raised up to 200 ± 30 ohms cm2 (Fig 4.3 e). It was observed that Vitamin D stimulated HaCaT cells monolayer protected from *S. aureus* secreted protease and also there was increased in TEER.

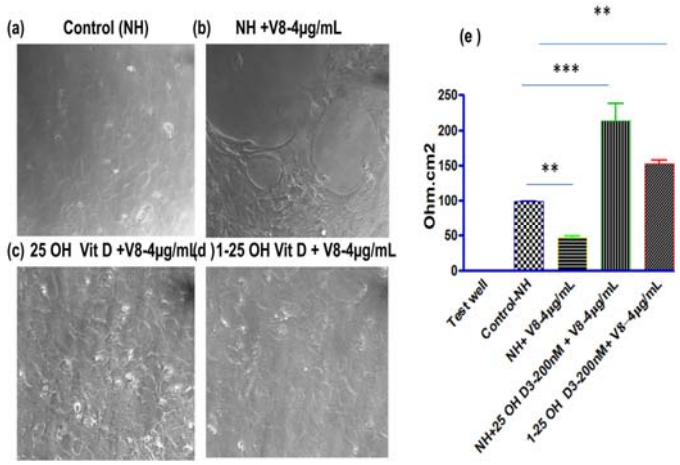


Figure 4.3 Vitamin D stimulated HaCaT cells protected from V8

HaCaT cells were grown on the Transwells with a mesh size of 0.45µm. (a) negative control, HaCaT cell monolayers not exposed to V8. (b) Positive control HaCaT cells exposed to 4 µg/mL of V8 for 24 hours. (c) HaCaT cell monolayers stimulated with 200 nM 25 OH Vitamin D3 for 24 hours then exposed to 4 µg/mL of V8 for another 24 hours. (d) HaCaT cell monolayers stimulated with 200nM 1, 25 OH Vitamin D3 for 24 hours, then exposed to 4 µg/mL of V8 for another 24 hours. (e) The difference in TEER of all the monolayers was measured with an ohm meter before and after the experiment. Data are representative of n=3 experiments and performed with replicates. (1 way ANOVA)

Tukey's Multiple Comparison Test

(Control-NH vs NH+ V8-4mg/mL P * < 0.05) (Control-NH vs NH+25 OH D3-200nM + V8-4mg/mL P **< 0.01) (Control-NH vs 1-25 OH D3-200nM+ V8-4mg/mL P * < 0.05)

4.2.4 Vitamin D3 and IL1-β/LTA- stimulated HaCaT cells' upregulate hBD2

In my previous experiments it has been shown that HaCaT cells were indirectly protected after stimulation with Vitamin D3 and IL1-β/LTA. It needs to find out the factor which is expressed after stimulation with Vitamin D3 and IL1-β/LTA. An ELISA was performed to examine the localization of the factor induced by HaCaT cells after stimulation with Vitamin D3 and IL1-β/LTA.

In this experiment, I used the following conditions: Wild type (Normal HaCaT cells) unstimulated and Wild type HaCaT cells stimulated with IL1-β/LTA and Vitamin D3 separately for 24 hours. All the cells, including unstimulated and stimulated were lysed after 24 hours. The concentration of hBD2 present in the lysate collected from unstimulated and IL1-β/LTA or Vitamin D-stimulated HaCaT cells, was analysed by sandwich ELISA. There was a difference between the hBD2 concentration which was found in the lysate collected from stimulated and unstimulated samples. The lysate obtained from unstimulated HaCaT cells contained 222 pg/mL of hBD2. IL1-β/LTA-stimulated HaCaT cell lysate contained 357 pg/ mL. Vitamin D3 stimulated HaCaT cell contained 728 pg/mL which is the highest concentration of hBD2 compare to other **(Fig 5.1)**. The ELISA results give us a simple association that there is more hBD2 secretion in the cells stimulated with Vitamin D3 and IL1-β/LTA.

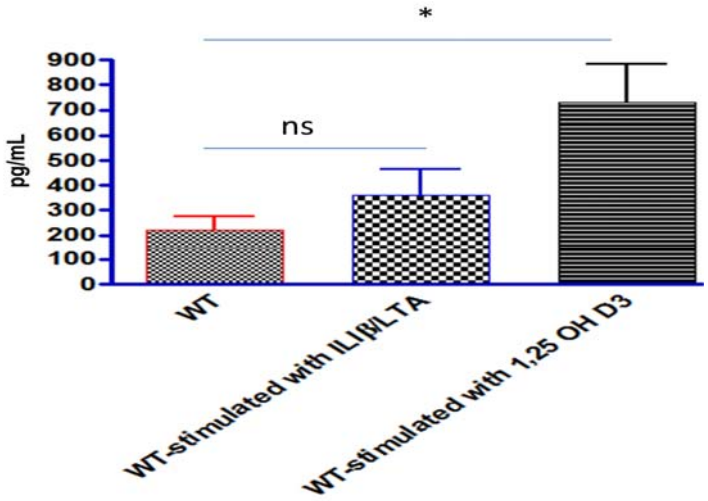


Figure 4.4: Increased hBD2 secretion by stimulating the cells detected by ELISA

Unstimulated normal HaCaT wild type (WT). Normal HaCaT cells were stimulated with (100ng+5µg) of IL1-β/LTA and (200 nM) of 1, 25 OH Vitamin D3 separately for 24 hours. ELISA was performed with the lysate from unstimulated WT HaCaT cells, IL1-β/LTA and 1, 25 OH Vitamin D3 stimulated cells. Data is representative of n=3 experiments and always performed with replicates (1 way ANOVA)

Tukey's Multiple Comparison Test: (WT vs WT-stimulated with ILIβ/LTA P > ns) (WT vs WT-stimulated with 1, 25 OH D3 P * < 0.05).

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4.2.5 Restoration of permeability barrier homeostasis with FITC-albumin

Thus to determine para- and transcellulr resistance separately, which cannot be achieved by conventional transepitlial resistance measurement **(See page 112)**. We used FITC-alb approach to optimize the effect of *S.aureus* proteases on the permeability barrier in HaCaT cell monolayer by using trancellular pathways. Artificial permeability barrier homeostasis was quantified by using FITC-alb fluorescence dye. FITC-alb dye transportation facilitated across the transcellular gaps produced by 4 and 8 µg/mL of V8 protease. **U**ntreated and HaCaT cell monolayer treated with hBD2 were exposed to V8 protease for 24 hours.The rate of FITC-albumin transference across the monolayer which was calculated in the sense of change in the florescence intensity and reading are presented here after deducting base reading of empty wells **(Fig 4.5 a).**

To differentiate the FITC-alb cleavage by V8 protease, percentage difference in the fluorescence intensity was calculated. Where Empty well + FITC-alb+ V8-8mg/mL refered as a 100 % having maximum increase in the in the fluorescence intensity **(Fig 4.5 b).** I observed there was not significant differences in the FITC-alb transference in the sense of change in the fluorescent intensity of untreated and treated HaCaT cell monolayes.

Figure- 4.5 FITC-albumin assay quantitative measure for HaCaT cell permeability. HaCaT cells grew on the 0.45μM pore-size transwell, with cell density of $2x10^5$ cells. (a) After 24 hours of exposure to monolayer with 4μg/mL and 8μg/mL was quantitated by the rate of FITC-albumin transference across the monolayer which was calculated in the sense of change in the fluorescence intensity. NH + 4μg/mL and 8μg/mL of V8, synthetic hBD2 (s hBD2), Cysteine-replaced-with-serine linear hBD2 (C-S), or scrambled hBD2 (SCB). Over-expressing hBD2 stable HaCaT cell monolayers, Empty well + FITC- alb, Empty well + FITC-alb+ V8-8mg/mL after 24 hours A.

(b) After 24 hours the percentage difference in the fluorescence intensity calculated where Empty well + FITC-alb+ V8-8mg/mL refered as a 100 % having maximum increase in the fluorescence intensity

Tukey's Multiple Comparison Test

(NH vs NH+V8-4mg/mL P > 0.05) (NH vs shBD2+V8-4mg/mL P > 0.05) (NH vs C-S-hBD2+V8-4mg/mL P > 0.05) (NH vs SCB-Peptide+V8-4mg/mL P<0.05)(NHvs hBD2OE+V8-4mg/mL P > 0.05) (NH vs NH+V8-8mg/mL P < 0.01) (NH vs shBD2+V8-8mg/mL P > 0.05) (NH vs C-S-hBD2+V8-8mg/mL P < 0.05) (NH vs SCB-Peptide+V8-8mg/mL P < 0.05) (NH vs hBD2OE+V8-8mg/mL P > 0.05) (NH vs EW-FIT-alb P > 0.05) (NH vs EW-FIT-V8-8mg/mL P < 0.01)

4.3 **Discussion:**

In addition to damaging the epidermal permeability barrier, *S. aureus* proteases aggravated the antimicrobial barrier by degrading adhesion junctions and tight junction proteins (**Amagai and Stanley, 2000).** After a series of experiments I observed that *S. aureus*-secreted proteases present in the supernatant could be the virulence factors which work on their specific targets against HaCaT cell monolayers as stated before by **Amagai.**

S. epidermidis is a skin commensal, known to secrete Lipid S which plays an important role during host bacterial infection by stimulating immune cells through TLR2. Lipid S induces up-regulation of skin antibacterial defense mechanisms, which protect against *S. aureus* infections **(Jones *et al.*, 2005).** I observed that HKSE-stimulated HaCaT cells were protected from the deleterious effect of the proteases present in the supernatant and V8 protease, while control unstimulated HaCaT cells were not protected. This protection of the HaCaT cell monolayer could be due to the secretory LTA (lipid S), which would expected to be present in the heat-killed *S. epidermidis* (HKSE) solution. I used recombinant *S.aureus* LTA, in order to testify the role *S.aureus* LTA in the protection of HaCaT cells from the deleterious effect of the proteases present in the supernatant and V8 protease. The common molecular architecture of LTA consists of a diacyleglycerol- containing glycolipid anchor and a covalently coupled polymeric backbone structure. However, LTA from different gram positive species have been found to differ in the chemical composition of the repeating units of the polymeric backbone. In *S.aureus*, the repeating units contain D-alanine and D-Nacetylglucosamine

linked to a central linear 1-3 linked polyglycerolphosphate chain **(Fischer W et al., 1994)** .*S.aureus* LTA by itself in multiple experiments did not provide good protection against *S. aureus* proteases (data not presented). Morath had shown that commercial LTA is of poor quality and purity and may not be biologically relevant **(Morath et al., 2002).** A study has shown that during *S.aureus* infection keratinocytes respond by secreating cationic peptide, which can interact with LTA **(Scott MG et al., 1999).** I observed that HaCaT cells exposed to LTA in the presence of recombinant IL1-β provides better protection against *S. aureus*-secreted proteases by expressing a secreted factor. IL1-β/LTA stimulated HaCaT cells were very well protected from deleterious effects of proteases present in the supernatant, and in addition this blocked the damaging effect of neat V8 protease (fig 4.2 page 155).

TLR2s are the key pathogen recognition receptors, correlated with *S. aureus* colonization, infection and increased disease severity due to modulation of the innate and adaptive responses **(Ahmad-Nejad *et al.*, 2004).** TLR2 is considered to be a critical ligand for host cell recognition of *S. aureus* and LTA, which alters the expression of pro-inflammatory mediators through activation of gene transcription factors such as NF-κB.

TLR2 single nucleotide polymorphism is found in 10% of AD patients, which can cause alteration of TLR2 function. Diminish, host signaling and host cell-pathogen interactions due to altered TLR2 surface ligands could result in the down regulation of antibacterial defense mechanism **(Lorenz *et al.*, 2000).** Individuals with AD carrying TLR2 mutations, an alternative ?pathway may be needed to protect the skin barrier,

vitamin D3 could play a role in this regard. Bikle had found that vitamin D3 (1, 25-D) is an important factor essential for normal keratinocyte development and function which plays a key role in the maintenance of skin barrier function in individuals with AD **(Bikle DD, *et al,* 2008).**

In addition, 1, 25-D has been suggested to increase innate immunity in skin and to enable efficient antimicrobial defines at epithelial surfaces **(Schauber J&Gallo RL, 2007).** Here, for the first time, we present a mechanistic insight into how 25-D and 1, 25-D can protect the skin barrier against *S. aureus*-secreted proteases by promoting expression of a protective factor. I found that 25-D and 1, 25-D-stimulated HaCaT cells were protected from the deleterious effects of V8 protease, whilst unstimulated HaCaT cells were not protected. In this experiment TEER measurement of the unstimulated and unexposed normal HaCaT cell monolayer was considered as a control. The TEER reading was dropped in the HaCaT cell monolayer exposed to 4µg/mL of V8 protease. Instead HaCaT cell monolayer resistant was increased when stimulating them with 25-D and 1, 25-D before exposing to 4µg/mL of V8 **(Fig 4.3 e).** Although 25-D and 1, 25-D share similar binding affinity for VDR but activation of VDR receptor is achieved by a delicate balance between the concentration of a number of endogenous hormones **(Marshall, T. G et al., 2008).**

Moreover, the antimicrobial barrier (AMB) and permeability barrier are linked because both water loss and pathogen invasion occur by the breakdown of intercellular junctions, present in the lipid matrix **(Borkowski *et al.,* 2011).** As previously mentioned in the chapter 1, protease and protease inhibitor balance plays a significant role in the

maintenance of barrier functions in AD. I tested the supportive role played by pro-inflammatory cytokine IL1-β in the presence of LTA which by secreting unknown factor protect, HaCaT cell monolayer from *S. aureus* secreted proteases. The factor which protects the HaCaT cell monolayer from *S.aureus* secreted protease by acting as a protease inhibitor could be used for the maintenance of barrier function in AD.

An ELISA was performed to examine the localization of factor induced by HaCaT cells after stimulation with Vitamin D3 and IL1-β/LTA **(Fig 4.4 page160).** The cationic nature of hBD2, possess its high affinity for charge polar head group of lipid. It may say, secreted hBD2 rapidly interact with extracellular lipid which cause coating of the plasma membrane with hBD2. I observed that by increasing stimulation time majority of hBD2 is cell-associated compared with its presence in the supernatant. At each time point supernatant and cell lysate was collected to analyse hBD2 by ELISA.

To assess, directly, the link between hBD2 expression and permeability barrier function, I restored permeability barrier homeostasis artificially, by growing HaCaT cells on a transwell membrane. In order to prevent alteration in permeability barrier I grew normal HaCaT cells in different wells. 90% confluent normal HaCaT cells were pretreated with 4 µg/mL of synthetic hBD2, C-S hBD2, SCB scrambled peptide and hBD2 over-expressing HaCaT cell-lines were exposed with 4 and 8 µg/mL of V8 protease. Next, 1 tested permeability barrier homeostasis, by growing hBD2 over-expressing HaCaT cell-lines on the transwell.

Artificial permeability barrier homeostasis was quantified by using FITC-alb fluorescence dye **(Wischke et al., 2005)**. FITC-alb dye transportation was facilitated across the transcellular gaps produced by 4 and 8 µg/mL of V8 protease. The rate of FITC-alb dye transportation across the disturbed barrier was quantitated by measuring OD with spectrophotometer after 24 hours **(Figure- 4.5).** Altogether, these data suggest that *S. aureus* proteases along with damaging the HaCaT cell monolayer also cause increased degradation of Fitc-alb. Using FITC-alb technique, it is not possible to accurately quantify structural damage to HaCaT cell monolayer, caused by *S. aureus* proteases.

4.4 Conclusion

I assessed the effect that IL1-β has, in the context of LTA, on the susceptibility of HaCaT cells to damage by *S.aureus* secreated proteases by upregulating hBD2.

S. epidermidis is a skin commensal that secrets lipid S a short chain length of cell wall LTA, thought to up regulate the expression and secretion of a peptide after binding to the toll-like receptor, TLR2. I used IL1-β has, in the context of LTA solution to stimulate HaCaT to upregulating hBD2 which protect monolayer from *S.aureus* proteases. In addition, Vitamin D-stimulated cells upregulating hBD2 in the CM which protects the monolayer. In order to identify that hBD2 is upregulated after stimulation with IL1-β/LTA, IL1-β, Vitamin D, I undertook further experiments.

5- **Identification of a factor produced by HaCaT cell having antiprotease action:**

5.1 Introduction

Atopic dermatitis patients are more prone to *S. aureus* infection, which could be due to the down-regulation of antibacterial defense **(Gambichler T *et al.*, 2008).** The data described in chapter 4 suggested that HaCaT cells expressed a protective factor when being stimulated with Vitamin D3, HKSE and IL1-β/LTA. That factor expressed by HaCaT cells protected the monolayer from the *S. aureus*-secreted proteases.

S.aureus cell wall components Peptidoglycan (PGN) and Lipoteichoic acid (LTA) play an important role during host bacterial infection by stimulating immune cells through TLR2. The PGN and LTA lead to the production of IL1-β which contributes host defense against invading microbes (**Morath *et al.*, 2001).** The Peptidoglycan (PGN) is a large polymer consisting of glycan strands of N-acetyl glucosamine and N-acetyl muramic acid that are cross-linked by Pentaglycine Bridge to form a 3-dimentional framework. The innate immune receptor stimulated specifically by PGN is still an issue of debate **(Inohara N., Nunez G 2005*).*** An interaction of *S.aureus* PGN with TLR2 has been suggested, but extensive purification of PGN eliminated TLR2 signaling **(Travassos L. H., Boneca I. G 2004*).*** Thus, it was concluded that LPP (lipopeptides) and other yet unidentified contaminants are responsible for PGN activity on TLR2 **(Inohara N., Nunez G 2005).** However, differences in the procedure used to isolate and purify PGN made it difficult to directly compare opposing results concerning the TLR2

activity of PGN. CD14 may act as a TLR co-receptor by interacting with *S.aureus* LTA, PGN and enhancing TLR2 activation. Since *S.aureus* LTA, PGN and Lipopeptides have distinctive biochemical structure; it was unclear how one receptor could recognize such a broad spectrum of molecules.

As mentioned before, defense against *S. aureus* can be promoted by antimicrobial peptides and proteins (e.g. defensins, elafin, SLPI and cathelicidin). Atopic dermatitis patients are more prone to *S. aureus* infection, which could be due to the down-regulation of antibacterial defense **(Gambichler T *et al.*, 2008).** In normal conditions, keratinocytes express very low levels of AMP, but during infection, inflammation, and wounding their expression is up-regulated and AMP accumulate in lamellar bodies of the stratum granulosum of the epidermis **(Gallo RL *et al.*, 2002).** There are different mechanisms described for the down-regulation of antimicrobial peptides (AMP). One study described how abnormal extrusion of the contents of the lamellar body in the dried skin areas of atopic dermatitis patients may lead to a permeability barrier abnormality **(Fartasc h M *et al.*, 1992).** Lai and Gallo said keratinocytes are the main source of antibacterial defense in normal human skin, but when skin becomes inflamed, recruited leukocytes contribute the majority of antimicrobial activity **(Lai Y and Gallo, 2009).**

It was also observed that amount of hBD2 secretion is different in different conditions. Psoriasis is an autoimmune disease with inflammatory condition in which patient have dry raised, red areas covered with silver color scaly patches appear on the skin. Researchers believe that for a person to develop psoriasis, the individual must have

a combination of the genes that cause psoriasis and be exposed to specific external factors known as "triggers" such as, stress, injury, medication and infection. A previous study described the presence of abundant AMP in the epidermis of all patients with psoriasis; prevent them from skin infection however these peptides were significantly decreased in the lesions from patients with AD **(Ong PY *et al.*, 2002).** Therefore, it is needed some external factor that can up-regulate skin anti-bacterial defense mechanisms, which protect against *S. aureus* infections, that external factor could be LTA **(Jones KJ *et al.*, 2005).**

The second most important factor that can cause reduce expression of hBD2 in atopic dermatitis patient is TLR2 mutation. There is evidence of TLR2 mutation in atopic dermatitis, which can cause alterations in TLR2 surface ligands that may diminish host signaling and host cell-pathogen interactions, resulting in the down-regulation of antimicrobial defense **(Lorenz E *et al.*, 2000)**.

The importance of Vitamin D has been observed when inflammation in individuals with AD was reduced with UVB radiation **(Bikle DD, *et al*, 2004)**. 1, 25 OH D3 regulates keratinocyte proliferation, differentiation and the formation of intact epidermal barrier. Alteration in local vitamin D3 concentration and or activation will likely affect normal cutaneous immune function, barrier function and inflammation **(Bikle DD, *et al*, 2004)**. Data presented in chapter 4 described how Vitamin D-stimulated HaCaT cells protected from *S. aureus*-secreted proteases **(Figure 4.3 158).** The AD patints having TLR2 mutation, vitamin D3 could be a fundamental external factor, require for hBD2 expression (cross reference). Synthesis of pre-vitamin D3 from 7-dehydrocholestrol occurs in the skin where narrow band (NB-UVB) radiation converts 7-

dehydrocholestrol into calcitriol, pre-vitamin D3. To form the active hormone, calcitriol must be hydroxylated twice to form calcidal (25 hydroxy vitamin D3, 25 D3) and finally active calcitriol (1, 25 dihydroxy vitamin D3, 1, 25 D3) **(Prosser DE, *et al.*, 2004).** Keratinocytes express two enzymes (CYP27A1) and (CYP27B1) that are capable of producing active 1, 25 OH D3 independent of renal and hepatic hydroxylation steps. The vitamin D3 is made in different layers of the epidermis, primarily in the stratum basale **(Bikle DD *et al*., 2008)**

Narrow band (NB-UVB) improves Vitamin D balance (described in the above paragraph) which alters antimicrobial peptide (AMP) expression in skin lesions of psoriasis patients and individuals with AD **(Vahavihu *et al.*, 2010).** It is known that, Vitamin D3 upregulate antimicrobial peptide expression. Though vitamin D3 has been suggested to increase innate immunity in skin and to enable efficient antimicrobial defense at epithelial surfaces **(Schauber J&Gallo RL, 2007).** Along with inducer of innate immunity Vitamin D3 is considered an important factor, essential for normal keratinocyte development and function **(Bikll DD, *et al,* 2008).**

After ELISA results, **(Figure 4.4 page 160)** we found there was significant increase in hBD2 secretion after stimulating with Vitamin D3. As a consequence of our ELISA results I planned to knock down hBD2 which expresses by HaCaT cells. The protective response by knocked down HaCaT cells **(Figure 5.3 page 179)** will be re-evaluated after stimulating with Vitamin D3, HKSE and IL1-β/LTA.

I hypothesized that the over-expression of antimicrobial peptide (AMP) may be an important factor which provides protection against *S.aureus* secreted proteases. To find out how HaCaT cell can be protect from the proteases in the presence or absence of AMP. I will assess the responses of HaCaT cell against *S.aureus* secreted proteases, when HaCaT cell will be knocking-down by siRNA using siRNA transfection **(Figure 5.3page 179)**. hBD2 overexpressing HaCaT cells stable line can be made by transfecting HaCaT cells with DEFB4 gene plasmid. For this purpose QRT-PCR will be performed for *DEFB4* gene expression which was normalised to 18S ribosomal RNA **(See page 114).** The protection against *S.aureus* proteases was tested when hBD2 overexpressing HaCaT cell lines exposed to proteases.

5.2 Results

5.2.1 Immunohistochemistry shows IL1-β/LTA up-regulate hBD2 expression.

To determine whether IL1-β/LTA stimulated HaCaT cells shows an increase in the expression and secretion of hBD2, Immunohistochemistry experiment was conducted (See Page # 104). HaCaT cells were seeded with a density of $2x10^5$ cells in chamber wells plates and grown to confluence, and werthen stimulated with IL1-β/LTA for 24 hours. Unstimulated and stimulated monolayers were fixed and stained with Phalloidin and DAPI, afterincubation with anti- hBD2 primary and appropriate secondary antibodies. Photographs were taken by confocal laser microscope where it appears the presence of hBD2 in the cytoplasm of.The unstimulated HaCaT cells have Perinuclear distribution of hBD2 **(Fig 5.1 a) while .** The perinuclear

distribution of hBD2 peptide in unstimulated control cells compared with IL1-β/LTA stimulated HaCaT cells. The morphological appearances of hBD2 induction in IL1-β/LTA-stimulated HaCaT cells were different from the unstimulated cells **(Fig 5.1 b).** Moreover, stimulated cells have punctate particles in the cytoplasm and on the outside of the membrane, but not condensed around the nucleus **(Fig 5.1 c)**. The immunostaining of perinuclearly distributed punctuate particles were suggestive of endoplasmic reticulum and Golgi apparatus localisation, which was a character of secreted, single peptide product. Realistically after viewing the photograph, it looks like hBD2 peptide disperses out from the stimulated cells into the cytoplasm. The secretory phase of hBD2 in the stimulated cells was followed by the phase where hBD2 was viewed as bound to the plasma membrane. These findings showed the secretory pattern of hBD2 peptide in the vast majority of HaCaT cells. I would say that hBD2 induction in HaCaT cells can be quantified in two different ways. First, induction of hBD2 peptide in unstimulated and stimulated HaCaT cells, which can be differentiated by their pattern of distribution. Second, both unstimulated and stimulated HaCaT cells can be quantified by counting the number of cells which express hBD2 induction **(Fig 5.1 d)**

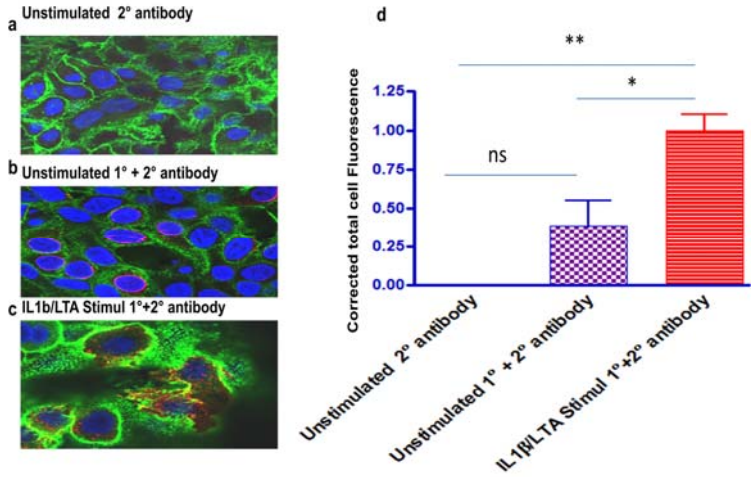


Figure-5.1 Immunohistochemistry to differentiate hBD2 secretion by IL1-β/LTA stimulation. HaCaT cells were incubated with primary antibody (1°) and appropriate secondary antibody (2°). Phalloidin and DAPI staining were also visible in that picture. (a) Unstimulated HaCaT cells were incubated with secondary antibody only. (b) Unstimulated HaCaT cells incubated with primary and appropriate secondary antibodies. (c) IL1-β/LTA-stimulated cells incubated with primary and appropriate secondary antibodies. (d) ImageJ quantification of the total cell fluorescence corrected by taking off the background revealed a prominent increase in corrected total cell fluorescence (CTCF) count, in IL1-β/LTA-stimulated HaCaT cells. Data is representative of n= 3 experiments and always performed with replicates (one way ANOVA). Magnification X400. **Tukey's Multiple Comparison Test:** (Control-NH vs NH-unstimulated $P > 0.05$) (Control-NH vs IL1-β/LTA-stimulated $P < 0.01$) (NH-unstimulated vs IL1-β/LTA -stimulated $P > 0.05$) (Tukey's Multiple Comparison Test)

5.2.2 Immunofluorescence analysis to visualize the extent of damage to Claudin-1 caused by *S.aureus*-secreted proteases-HaCaT cells were seeded with a density of $2x10^5$ cells/350µL in the chamber wells plate and grown to confluence. HaCaT cell monolayer exposed with 4 and 8µg/mL of V8 for twenty four hours. Unexposed and exposed monolayer's were fixed after 24 hours and then incubated with appropriate primary and secondary antibodies. HaCaT cell monolayer in the chambers wells plate was stained with phalloidin-FITC and DAPI to evaluate changes in the epidermal cytoskeleton. Photographs were taken by confocal laser microscope. Immunostaining **(See page # 105)** at the cell-cell borders was reduced when HaCaT cells were exposed to 4 and 8 µg/mL of V8 for 24 hours. Control unexposed HaCaT cell monolayer's did not show breakage of the cell cytoskeleton when looked with confocal laser microscope **(Fig 5.2 a).** HaCaT cell monolayer's exposed to 4 µg/mL of V8, which causes cells to retract from each other when tight junctions were broken, ultimately it make holes formation in the monolayer **(Fig 5.2 b).** There was significant damage to the HaCaT cell monolayer with 8 µg/mL of V8 after 24 hours of exposure which results in bigger holes formation in the monolayer **(Fig 5.2 c).** The percentage damage to HaCaT cell monolayer when exposed with 4 and 8µg/mL of V8 was calculated by using Image J software **(Fig 5.2 d).** There was no damage to unexposed normal HaCaT cell monolayer. The HaCaT cell monolayer exposed to 4µg/mL of V8 had 25 % damage and HaCaT cell monolayer exposed to 8µg/mL of V8 had 40% damage compare to control. The damage to the monolayer resulted in the loss of cells at that area and creates gaps in HaCaT cell monolayer. Therefore due to loss of cells in the damaged area of monolayer, there will be no claudin expression which results in the reduction of CLDN-1 staining.

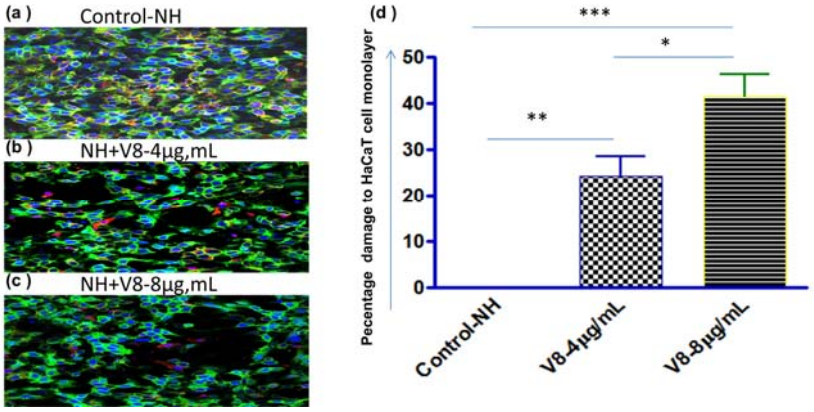


Figure 5.2 Immunofluorescence analysis after exposing HaCaT cell monolayer to V8 protease-HaCaT cell monolayer exposed with 4 and 8µg/mL of V8 for twenty four hours. Unexposed and exposed monolayer's were fixed after 24 hours and then incubated with appropriate primary and secondary antibodies. All the chambers were stained with phalloidin-FITC and DAPI to evaluate changes in the HaCaT cell monolayer cytoskeleton. **(a)** Control normal unexposed HaCaT cells stained with primary claudin-1 antibody and secondary antibody (Control-NH; n=4). **(b)** Normal HaCaT cells exposed to 4µg/mL of V8 (V8-4µg/mL; n=4). **(c)** Normal HaCaT cells exposed to 8µg/mL of V8 (V8-8µg/mL; n=4). **(d)** Image J quantification of the percentage damaged caused by exposing normal HaCaT cells with 4 and 8µg/mL of V8. Data are shown as mean fold change (+/- SEM) over unexposed cells magnification X200. (One way ANOVA)

Tukey's Multiple Comparison Test

(Control-NH vs NH+V8-4µg/mL $P < 0.01$) (Control-NH vs NH+V8-8µg/mL $P < 0.001$) (NH+V8-4µg/mL vs NH+V8-8µg/mL $P < 0.05$)

5.2.3 hBD2 siRNA knockdown ablates the protective response in HaCaT cells

I hypothesized that hBD2 was the key protective factor released in response to IL1-β/ LTA stimulation of HaCaT cells. Sandwich ELISA **(Ssee page # 100)** confirmed that hBD2 was expressed by the HaCaT cells with IL1-β/ LTA and Vitamin D3 stimulation. In order to determine the protective effect of hBD2, it was knocked it down with hBD2-siRNA (cross ref). For the knock-down assay, HaCaT cells were transfected with control and hBD2 -siRNA. After two days of transfection, HaCaT cell monolayers were exposed to 4µg/mL of V8 protease for twenty four hours. In the absence of IL-1 β/ LTA stimulation, both control and hBD2 -siRNA transfected HaCaT cells were unable to protect the monolayer from the deleterious effect of V8 **(Fig 5.3 a-c)**. Control and hBD2 -siRNA transfected HaCaT cells were stimulated with (IL1-β 100ng/ LTA 5µg) in 1 mL of culture medium for 24 hours, and then exposed with 4µg/mL of V8 for another 24 hours. Control siRNA transfected HaCaT cells stimulated with IL1-β/LTA protect the monolayer from the deleterious effect of V8 **(Fig 5.3 b).** However, hBD2 siRNA blocked the protection afforded by IL1-β/LTA stimulation **(Fig 5.3 d).** Demonstrating that hBD2 expression in response to these stimuli was required for the protective phenotype. Percentage damage caused by V8 protease calculated by image J software which is the (mean +/- SD) **(Fig 5.3 e).** Where (control siRNA +V8) has 15 % damage, (hBD2 siRNA+V8) has 20 %, (control siRNA + IL1-β/LTA +V8) has no damage normal looking monolayer, (hBD2 siRNA+ IL1-β/LTA +V8) has 18% damage.

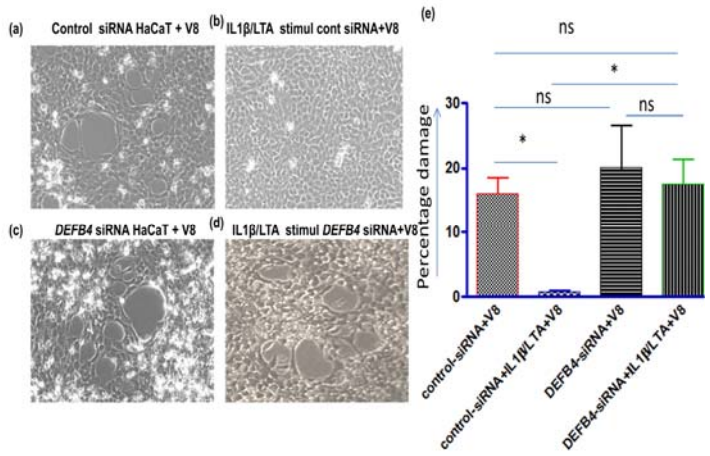


Figure 5.3: hBD2 siRNA transfected HaCaT cells exposed with V8 protease. Freshly seeded HaCaT cells were transfected with control and hBD2 -siRNA separately. 90% confluent transfected HaCaT cells were exposed to 4 µg/mL of V8 for 24 hours. DIC microscopic images revealed (a) Control siRNA transfected HaCaT cells was exposed to 4µg/mL of V8 protease for 24 hours. (b) IL1-β/LTA stimulated, control siRNA transfected HaCaT cells was exposed to 4µg/mL of V8 protease for 24 hours. (c) hBD2 siRNA transfected HaCaT cells monolayer was exposed to 4µg/mL of V8 protease for 24 hours. (d) IL1-β/LTA stimulated hBD2 siRNA transfected HaCaT cells monolayer was exposed to 4µg/mL of V8 protease for 24 hours. (e) Size of the holes revealed the extent of damage to the HaCaT cell monolayer, percentage damage to the monolayer was calculated by using Image J software. Data are representative of n=3 experiments and always performed with replicates.(1 way ANOVA) Magnification X200.

Tukey's Multiple Comparison Post test; (Control-siRNA+V8vs Control-siRNA+IL1b/LTA+V8 P<0.05) (Control-siRNA+V8 vs DEFB4-siRNA+V8 P = ns) (Control-siRNA+V8 vs

DEFB4-siRNA+IL1b/LTA+V8P = ns) (Control siRNA+IL1b/LTA+V8vsDEFB4-siRNA+IL1b/LTA+V8 P > 0.05)

5.2.4 hBD2-over-expressed HaCaT cells are protected of 8325-4 and V8 protease - In order to further explore the role of hBD2 in protecting HaCaT cells from *S. aureus* proteases, I assessed the responses of HaCaT cell lines that over-expressed hBD2 **(See page # 101)**. The wild type normal HaCaT cells monolayers (**Figure 5.5 a).** The wild type normal HaCaT cells monolayer exposed to 0.04 DF supernatant of 8325-4 for 12 hours **(Figure 5.5 b)** and HaCaT cells over-expressing hBD2 monolayers were exposed to 0.04 DF supernatant of 8325-4 for 12 hours **(Figure 5.5 c).** Despite the protective effects afforded by over-expression of hBD2 against the proteases in the supernatant, I tested the protective effect against recombinant V8. The wild type normal HaCaT cells monolayers and HaCaT cells over-expressing hBD2 were exposed to 4-µg/mL of V8 for 24 hours. The wild type normal HaCaT cells monolayers were unable to protect from V8 and there were holes formation through the monolayer **(Fig 5.5 d).** whilst hBD2 over-expressing HaCaT cells were protected from V8 protease **(Fig 5.5 e)** percentage damage to the HaCaT cell monolayer by 0.04 DF supernatant of 8325-4 and recombinant V8 protease was quantitated with ImageJ software **(Fig 5.5 f)** (WT- Normal HaCaT +0.04 DF supernatant of 8325-4 for 12 hours has 25% damage) (hBD2 over-expressing HaCaT cell monolayers + 0.04 DF supernatant of 8325-4 for12 hours has 7% damage) (WT-Normal HaCaT cell monolayers+ 4-µg/mL of V8 for 24 hours has 30% damage) (hBD2 over-expressing HaCaT cell monolayers + 4-µg/mL of V8 for 24 hours has 5 % damage) **(Fig 5.5 f)** The wild type normal HaCaT cells monolayers were not able to block the effect of proteases present in the 0.04 DF supernatant of 8325-4, whilst over-expressing hBD2 monolayers almost completely blocked the effect of proteases present in the 0.04 DF supernatant of 8325-4.

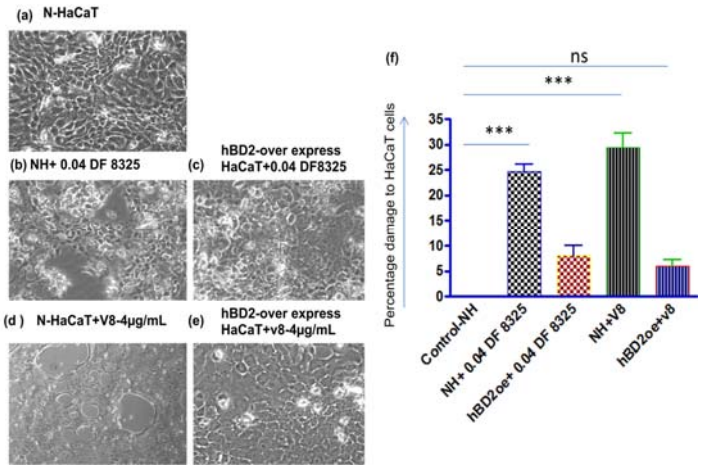


Figure 5.4 hBD2 **over-expressing HaCaT cells exposed to 8325-4 CM and V8**

DIC microscopic images revealed (a) unexposed, wild type (WT) normal HaCaT cells. (b) WT- Normal HaCaT cell monolayers were exposed to 0.04 DF supernatant of 8325-4 for 12 hours (c) hBD2 over-expressing HaCaT cell monolayers were exposed to 0.04 DF supernatant of 8325-4 for12 hours. (d) WT-Normal HaCaT cell monolayers were exposed to 4-µg/mL of V8 for 24 hours. (e) hBD2 over-expressing HaCaT cell monolayers were exposed to 4-µg/mL of V8 for 24 hours (f) percentage damage to the HaCaT cell monolayer by 0.04 DF supernatant of 8325-4 and V8 protease was quantitated with ImageJ software. Data are representative of n=3 experiments and always performed with replicates at Magnification X400 (One way ANOVA) **Tukey's Multiple Comparison Test-**(Control-NHvsNH+0.04DF-8325-12hP<0.001)(Control-NHvshBD2-overexpress HaCaT+0.04DF-8325-4 P>0.05)(Control-NH vs NH+V8-4mg/mLP<0.001)(Control-NH vs hBD2-over express HaCaT+V8-4mgP > 0.05).

5.2.5 - *DEFB4* gene expression in hBD2 or hBD3 over-expressing HaCaT cell lines and wild type HaCaT cells when stimulated with IL1-β

As mention before control and hBD2-siRNA transfected HaCaT cells were unable to protect the monolayer from the deleterious effect of V8 **(Fig 5.3 a-b)**. When control and hBD2-siRNA transfected HaCaT cells were stimulated with (IL1-β/ LTA) and then exposing with V8, only control siRNA transfected HaCaT cells stimulated with IL1-β/LTA was protective.The wild type normal HaCaT cells monolayers were unable to block the effect of proteases, whilst over-expressing hBD2 monolayers almost completely blocked the effect of proteases present in the supernatant of 8325-4. To further investigate this, whether hBD2 peptide encoded by *DEFB4* gene expression varied in stimulated and unstimulated HaCaT cells as well as in hBD2 over-expressing HaCaT cells QRT-PCR was performed. Source, Homo sapiens defensin, (DEFB4) mRNA, NCBI reference sequence: NM 004942, with size of 336 bp mRNA linear PRI. Version NM004942.2 GI; 13124885, keyword Refseq. For this purpose *DEFB4* gene expression was normalised to 18S ribosomal RNA. RNA was prepared from untreated HaCaT cells and cells stimulated with IL-1β 100 ng/ml for 48 hours. RNA was also prepared from HaCaT cells stable line made by transfected with DEFB4 gene plasmid to over express hBD2.The hBD3 over-expressing HaCaT stable cell line was made by transfecting with DEFB103 gene plasmid to overexpress hBD3. We observed that the hBD2 over-expressing cell-line expressed *DEFB4* gene which was higher than the IL-1β stimulated HaCaT cells **(Fig 5.6)**, these results are consistent with the changes in the expression of *DEFB4* gene in unstimulated and stimulated cells.

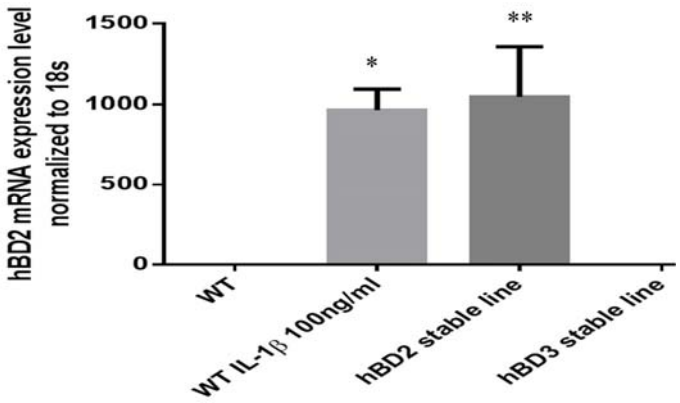


Figure 5.5 Assessment of *DEFB4* transcription in HaCaT cells

RNA was prepared from untreated HaCaT cells (WT; n=3), HaCaT cells stimulated with IL-1β 100 ng/ml for 48 hours (WT IL-1 β 100 ng/ml; n=3), HaCaT cells transfected with a *DEFB4* gene plasmid to overexpress hBD2 (hBD2 stable line; n=3) and HaCaT cells transfected with a *DEFB103* gene plasmid to overexpress hBD3 (hBD3 stable line; n=1). QRT-PCR was performed for *DEFB4* expression and normalised to 18S ribosomal RNA. Data are shown as mean fold change (+/- SEM) over untreated cells. $p < 0.01$, $p< 0.001$. (These data result from experiments conducted by Bingjie Wang and Dr Brian McHugh).

5.2.6 - Synthetic hBD2 treated HaCaT cells are protected from supernatant of 8325-4 and V8 protease

The hBD2 knock-down assay provided me thoughts that hBD2 could be the protective factor of the Vitamin D3, HKSE and IL1-β/LTA stimulated cells. To further test the protective action of hBD2, I performed the following series of experiments with synthetic linear and nonlinear hBD2. HaCaT cell monolayers wild type untreated **(Fig 5.4 a).** HaCaT cell monolayers (in serum free medium SFM) were treated with 4-µg/mL of synthetic hBD2 - two hours prior to exposing cells to 0.04 DF supernatant (0.04 dilution factor) of 8325-4 for 12 hours. Positive control untreated HaCaT cell monolayers were unable to protect themselves from the damaging effects caused by the supernatant of 8325-4 after 12 hours. Light microscope examination revealed the presence of voids and fissures in the monolayers **(Fig 5.4 b).** Two hours pre-treated HaCaT cell monolayers with synthetic hBD2 were protected from the supernatant of 8325-4 **(Fig 5.4 c).** Despite the protective effects afforded by synthetic hBD2 against the proteases in the supernatant, I tested the protective effect against recombinant V8. Untreated cells exposed to 4-µg/mL of V8 revealed formation of holes after 24 hours **(Fig 5.4 d).** Two hours pre-treated HaCaT cell monolayer with synthetic linear and non-linear hBD2 were protected from 4-µg/mL of V8 after 24 hours of exposure **(Fig 5.4 e-f).** ImageJ software was used to calculate damage to HaCaT cell monolayer with *S.aureus* proteases and then Percentage damage was obtained by comparing reading to unexposed which had 0% damage. Where (NH+ 0.04 DF supernatant (0.04 dilution factor) of 8325-4) has 25% damage to the monolayer, (NH+4-µg/mL of synthetic hBD2+ 0.04 DF(0.04 dilution factor) supernatant

of 8325-4) has 5 % damage, (NH+ 4-µg/mL of V8 after 24 hours) has 30% damage, (NH+4-µg/mL of synthetic hBD2+ 4-µg/mL of V8 after 24 hours) has 2% damage, (NH+4-µg/mL of C-S hBD2+ 4-µg/mL of V8 after 24 hours) has 3% damage **(Fig 5.4 g).**

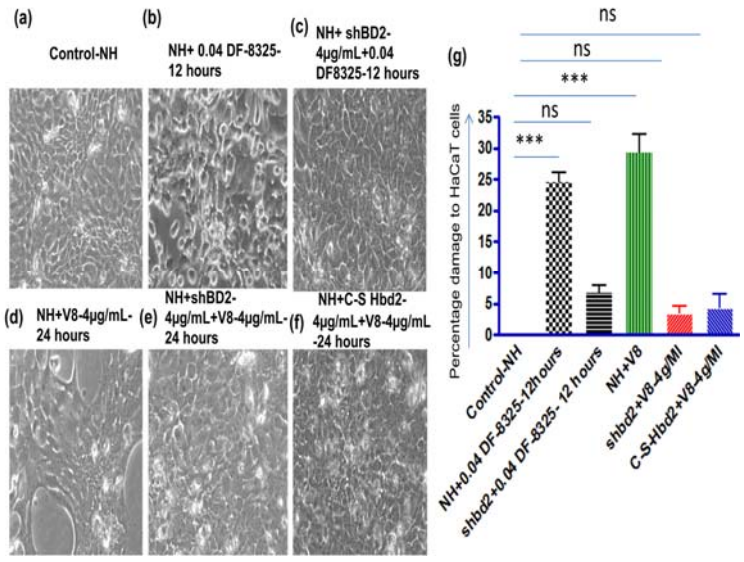


Figure 5.6 Synthetic hBD2 pre-treated HaCaT cells exposed to 8325-4,V8 protease

DIC microscopic images revealed **(a)** Normal HaCaT monolayer without any exposure. **(b)** Normal HaCaT monolayer exposed to supernatant of 8325-4. **(c)** 4-µg/mL of synthetic hBD2 pre-treated normal HaCaT monolayer was exposed to supernatant of 8325-4**. (d)** Normal HaCaT monolayer exposed to 4-µg/mL of V8 protease for 24 hours **(e)** 4-µg/mL of synthetic hBD2 pre-treated normal HaCaT monolayer was exposed to 4-µg/mL of V8 protease for 24 hours. **(f)** 4-µg/mL linear cysteine replaced with serine (C-S) hBD2 pre-treated normal HaCaT monolayer was exposed to 4-µg/mL of V8 protease for 24 hours **(g)** Percentage damage to HaCaT cell monolayer was calculated by using ImageJ software, Data are representative of n=4 experiments and always performed with

replicates at Magnification X400. (1 way ANOVA) **Tukey's Multiple Comparison Test :** (Control-NH vs NH+0.04DF-832-12 hours $P < 0.001$) (Control-NH vs ShBDd2-4mg/mL+0.04DF-8325-12 hours $P > 0.05$ =ns) (Control-NH vs NH+V8-4mg/mL-24 hours $P < 0.001$) (Control-NH vs Shbd2+V8-4mg/mL-24 hours $P > 0.05$=ns)(Control-NH vs C-S-hBD2-4mg/mL +V8-4mg/mL-24 hours $P > 0.05$=ns).

5.2.7 Western.Blot analysis for the cleavage of TJ protein Claudin-1 by *S.aureus* secreted proteases

Western. Blot analysis was performed to quantitate the cleavage of TJ protein Claudin-1 by proteases. HaCaT cells are seeded at a density of $4x10^4$ cells/cm^2, 90% confluent HaCaT cells monolayer was exposed with V8-2, 4, and 8µg/mL and 0.04 DF supernatant of 8325-4 for 12 hours. In another experiment HaCaT cell monolayers was exposed with V8-2, 4, and 8µg/mL for 24 hours. The extent of damage to the monolayer caused by V8 was visualised by light microscope before lysing the cells. Panactin protein was used as a loading control antibody and anti-claudin antibody used for claudin-1 protein. Electrophoretically transferred protein bands were visualized and photographed by using Liquor Odyssey **(Fig 5.7 a).**

The reduction in the band density was calculated by using Image J software. Where data are shown reduction in the band density compare to standardised loading control (Panactin protein) presented as percentage damage to the HaCaT cell monolayers **(Fig 5.7 b).**

(Panactin -V8-2µg/mL) after 12 hours no reduction, while 4% reduction in the band density was seen after 24 hours. (Panactin -V8-4µg/mL) after 12 hours 12 % reduction and after 24 hours (50%). (Panactin-V8-8µg/mL) 48% after 12 hours, 65% after 24 hours. (Panactin - 0.04 DF supernatant of 8325-4) 68% after 12 hours reduction in the band density. Data are shown as mean fold change (+/- SD) over standardised loading control (Panactin protein).Data are representative of experiments n=4 performed in replicates.

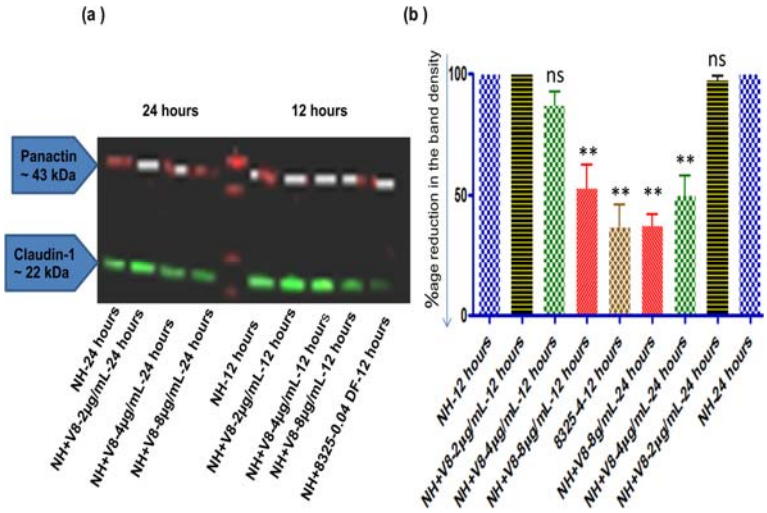


Figure 5.7 *S.aureus* serine proteases effects on Claudin-1band density.

Western Blot analysis was performed to quantitate the cleavage of TJ protein (Claudin-1) and extent of damage to the HaCaT cell monolayer by *S.aureus* proteases evaluated by comparing it with the loading control. (a) Unexposed normal HaCaT cells lysate collected after 12 hours (NH-12 hours; n=4), Normal HaCaT cells exposed to V8-2µg/mL lysate collected after 12 hours (NH+V8-2µg/mL-12 hours; n=4), Normal HaCaT cells exposed to V8-4µg/mL lysate collected after 12 hours (NH+V8-4µg/mL-12 hours; n=4), Normal HaCaT cells exposed to V8-8µg/mL lysate collected after12 hours (NH+V8-8µg/mL-12hours; n=4), Normal HaCaT cells exposed to 0.04 DF supernatant of 8325-4 lysate collected after 12 hours (NH+ 0.04 DF 8325-12 hours;

n=4). Unexposed normal HaCaT cells lysate collected after 24hours (NH-24hours; n=4), Normal HaCaT cells exposed to

V8-2µg/mL lysate collected after 24 hours (NH+V8-2µg/mL-24hours; n=4), Normal HaCaT cells exposed to V8-4µg/mL lysate collected after 24 hours (NH+V8-4µg/mL-24 hours; n=4), Normal HaCaT cells exposed to V8-8µg/mL lysate collected after24 hours (NH+V8-8µg/mL-24 hours; n=4). (b) The reduction in the band density was calculated by using Image J software. Data are shown as mean fold change (± SEM) over standardised loading control Panactin cells (One way ANOVA).

Tukey's Multiple Comparison Test

(Panactin-12 hours vs NH-12 hours P > 0.05) (Panactin-12 hour's vs NH+V8-2mg/mL-12 hours P > 0.05) (Panactin-12 hours vs NH+V8-4mg/mL-12 hours P > 0.05) (Panactin-12 hour's vs NH+V8-8mg/mL-12 hoursP< 0.001) (Panactin-12 hour's vs 8325-4-12 hoursP < 0.001) (Panactin-12 hour's vs NH+V8-8g/mL-24 hours P < 0.001) (Panactin-12 hour's vs NH+V8-4mg/mL-24 hours P < 0.001) (Panactin-12 hours vs NH+V8-2mg/mL-24 hours P > 0.

5.2.8 Determine the protection offered by stimulated and hBD2 stable HaCaT cell line against *S.aureus* secreted proteases by Western.Blot analysis

S. aureus-secreted proteases damaging affect to the HaCaT cells monolayer could be due to the cleavage of claudin-1 protein. The damage to HaCaT cells monolayer can be protected by stimulating HaCaT cells with IL1-β/LTA and with HaCaT stable cell-line which over-expressed hBD2 (hBD2OE). W.B analysis was performed to testify the protection offered by HaCaT cells stimulated with IL1-β/LTA and HaCaT stable cell-line. Lysates were collected and W.B analysis was performed by using claudin-1 primary antibody and Panactin was used as a standardised loading control **(Fig 5.8 a).**

The reduction of band intensity compared to standardised loading control Panactin was calculated by using Image J software. Where data are shown reduction in the band density presented as percentage damage to the monolayer (Fig **5.8** b). Panactin - V8-8µg/mL12 hours (50% reduction), Panactin - IL1-β/LTA+V8-8µg/mL after 12 hours (20% reduction in the band density). hBD2OE + V8-8µg/mL12 hours (12% reduction in the band density). Panactin – 0.04 DF supernatant of 8325-4 12 hours (80% reduction), Panactin - IL1-β/LTA+ 0.04 DF supernatant of 8325-4 12 hours (10 %). hBD2OE + 0.04 DF supernatant of 8325-412 hours (40% reduction). The protection was achieved when HaCaT cells monolayers was pre-stimulated with IL1-β/LTA (100 ng/mL/ 5µg/mL) and hBD2 expressed HaCaT cells were exposed to 8µg/mL of V8 and 0.04 DF supernatant of 8325-4 for 12 hours.

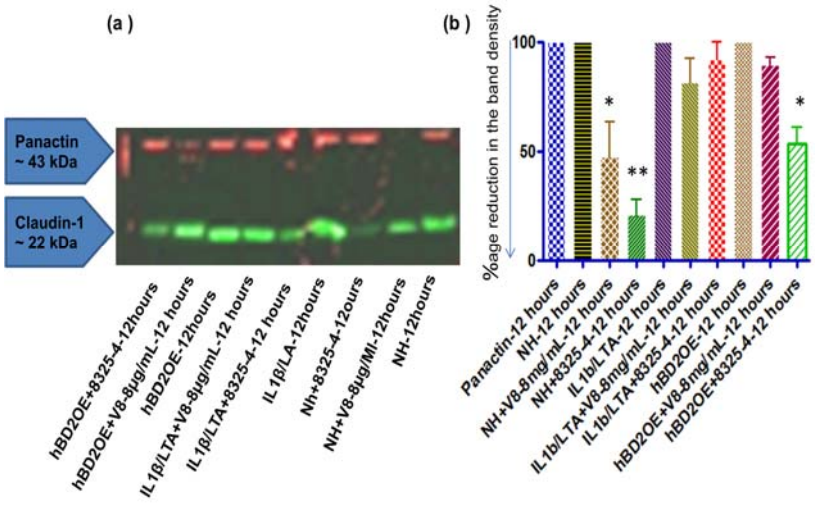


Figure 5.8 Stimulated & hBD2 overexpressing HaCaT cells exposed to V8 and 8325-4 12h.

Lysates were collected and W.B analysis was performed by using claudin-1 primary antibody and Panactin was used as a standardised loading control. (a) Unexposed normal HaCaT cells lysate collected after 12 hours (NH-12 hours; n=4), Normal HaCaT cells exposed to V8-8 µg/mL 12 hours (NH+V8-8µg/mL12 hours; n=4), Normal HaCaT cells exposed to 8325-4 12 hours (NH+V8-8µg/mL12 hours; n=4), HaCaT cells stimulated with IL1-β/LTA and lysate collected after12 hours (IL1-β/LTA-12 hours; n=4), HaCaT cells stimulated with IL1-β/LTA exposed to V8-8 µg/mL 12 hours, lysate collected after12 hours (IL1-β/LTA + V8-8 µg/mL 12 hours; n=4). IL1-β/LTA stimulated HaCaT cells exposed to 8325-4 12 hours (IL1-β/LTA + 8325-4 12 hours; n=4). HBD2 overexpressed HaCaT cells stable cell line lysate collected after 12 hours (hBD2OE-12

hours; n=4). hBD2 overexpressed HaCaT cells stable cell line exposed to V8-8 µg/mL 12 hours, lysate collected after12 hours (hBD2OE + V8-8 µg/mL 12 hours; n=4). hBD2 overexpressed HaCaT cells stable cell line exposed to 8325-4 12 hours (hBD2OE+8325-4 12 hours; n=4). (b) Western Blot analysis was performed with lysate collected after 12 hours and reduction in the Claudin protein band intensity was calculated by using Image J software. Data are shown reduction in the band intensity compare to standardised loading control Panactin. Data are representative of experiments n=4 and always performed in replicates (One way ANOVA).

Tukey's Multiple Comparison Test

(Panactin-12 hours vs NH-12 hours $P > 0.05$) (Panactin-12 hour's vs NH+V8-8mg/mL-12 hours $P < 0.01$) (Panactin-12 hour's vs NH+8325-4-12 hours $P < 0.001$) (Panactin-12 hour's vs IL1b/LTA-12 hours $P > 0.05$) (Panactin-12 hour's vs IL1b/LTA+V8-8mg/mL-12 hours $P > 0.05$) (Panactin-12 hour's vs IL1b/LTA+8325-4-12 hours $P > 0.05$) (Panactin-12 hours vs hBD2OE-12 hours $P>0.05$) (Panactin-12 hours vs hBD2OE+V8-8mg/mL-12 hours $P > 0.05$)**.**

5.3 Discussion

Atopic dermatitis patients are more prone to *S. aureus* infections, a phenotype that could be due to the down regulation of antibacterial defence **(Gambichler *et al.*, 2008)**. In normal conditions, keratinocytes express very low quantities of hBD2, but during infection, inflammation and wounding its expression is up-regulated and it accumulates in the lamellar bodies of the stratum granulosum layer of the epidermis **(Gallo *et al.*, 2002)**. Rie Ommori described how *S. aureus* and *S. epidermidis* secrete LTA, differentially, to induce subtypes of antimicrobial peptide (AMP) from human keratinocytes, through different receptors. Therefore, it is possible that *S. epidermidis* secretes (Lipid S) short chain LTA which after binding to TLR2 up-regulate skin antibacterial defence mechanisms, which protects against *S. aureus* infections **(Jones KJ*et al.*, 2005)**. It was mentioned before that TLR2 mutation modifies cytokine production and toll-like receptor expression, which may crucially modulate the pathogenesis of atopic dermatitis. More than 10% of patients with AD are heterozygous for TLR-2, R753Q single nucleotide polymorphism (SNP) **(Mrabet-Dhabi *et al.*, 2008).**

A previous study by Ong *et al.* confirmed the presence of abundant LL37 and hBD2 in the superficial epidermis of all patients with psoriasis. In contrast, significantly lesser amount of these peptides was present in acute and chronic lesions from patients with atopic dermatitis **(Ong *et al.*, 2002)**. LL37 having antimicrobial activity against bacteria, viruses, and fungi, is produced by epidermal keratinocytes, Reduced expression of antimicrobial peptides may cause recurrent infections in AD **(Mallbris L et al., 2010).** Vitamin D3 enhances LL37 production in keratinocytes, because vitamin D response

element (VDRE) occurs on the gene of LL-37 precursor cathelicidin. Oral vitamin D3 increases cathelicidin expression in AD lesions, however vitamin D role in pathogenesis of AD is controversial **(Hata TR., 2008) (Benson AA et al., 2012).** Vitamin D3 supplementation improves AD symptoms while vitamin D3 intake during infancy accelerates the development of AD **(Back O et al., 2009).** Studies have indicated that dysregulation of VDR may lead to exaggerated inflammatory responses, raising possibility that defect in vitamin D and VDR signaling transduction may be linked to bacterial infection and chronic inflammation **(Sun J et al., 2010).** The bacteria secrete Capnine, a protein which may bind to and inactivate the VDR. A growing number of substances and species have been shown to down-regulate the activity of the VDR. It is shown that there is down-regulation of VDR receptor by mycobacterium tuberculosis **(Xu Y et al., 2003)**.

The data described in chapter 4 suggested that HaCaT cells expressed a factor after being stimulated with Vitamin D3, HKSE and IL1-β/LTA, protected the monolayer from damage induced by the *S. aureus*-secreted proteases. Based on our ELISA results, I found that hBD2 could be that protective factor, which released in response to Vitamin D3, HKSE and IL1-β/LTA stimulation. In order to check the antiprotease action of hBD2, we knocked it down with hBD2 siRNA.

In order to see the direct role of hBD2 as a protease inhibitor, we knocked it down with hBD2 siRNA and quantified our results by calculating percentage damage to the monolayer caused by the protease. Not surprisingly, cells that had not been stimulated to make hBD2 had a similar phenotype whether they knocked down with siRNA or not.

We observed that hBD2-siRNA knock-down in HaCaT cells blocked the protection afforded by IL1-β/LTA stimulation, whilst control-siRNA knock-down HaCaT cells cannot block the protection afforded by IL1-β/LTA stimulation. To confirm increase secretion of hBD2 when stimulated with Vitamin D3, HKSE and IL1-β/LTA, we knocked down hBD2 in HaCaT cells with siRNA. We quantified our results by calculating percentage damaged to the monolayer caused by the protease. Not surprisingly, cells that had not been stimulated to make hBD2 had a similar phenotype whether they knocked down by siRNA or not. We observed that *DEFB4*-siRNA knock-down HaCaT cells blocked the protection afforded by IL1-β/LTA stimulation, whilst control-siRNA knock-down HaCaT cells cannot block the projection afforded by IL1-β/LTA stimulation. In order to clearly assess the impact of hBD2 expression by HaCaT Cells, I assessed the responses of stable hBD2 over-expressing HaCaT cell lines. Would they be protected from the *S. aureus*-secreted proteases? I found that, hBD2 over-expressing HaCaT cell lines protected from the damaging effect of V8 proteases.

In order to clearly assess the impact of hBD2 expression by HaCaT cells, I assessed the responses of stable hBD2 overexpressing HaCaT cell lines. Would they be protected from the *S. aureus*-secreted proteases? We found that, hBD2 overexpressing HaCaT cell lines protected from the damaging effect of V8 at concentrations of 4-µg/mL. After our hBD2 knockdown and hBD2 overexpression models worked very well, I tried to see the effect of synthetic hBD2 peptides. It was found that synthetic peptides work as well to protect the monolayer from the damaging effect of *S.aureus* secreted proteases.

5.4 Conclusion- hBD2 knockdown cells were unable to protect the monolayer, whilst cells overexpressing hBD2 were able to protect the HaCaT cell monolayers from the proteases in the supernatant, or recombinant V8 (serine) proteases. Synthetic hBD2 could also act as exogenously applied protective factor. The mechanism of anti-protease action of hBD2 remains unclear, further study need to find out the chemical interaction of hBD2 with *S.aureus* protease for the protection of HaCaT cell monolayer either it work in-directly or it has direct anti-protease activity?

6-Protease inhibitor action of hBD2: Characterised by enzyme kinetic assay.

6.1 Introduction

HaCaT cells transfected with hBD2 siRNA were unable to protect from the destructive potential of *S.aureus* proteases, while hBD2 over-expressing HaCaT cells were protected from the *S.aureus* proteases. This observation raised the possibility that hBD2 inhibits V8 protease by having anti-protease action. The anti-protease effect of hBD2 was tested by protecting HaCaT cell monolayers against *S. aureus* secreted proteases in the supernatant of strain 8325-4, RN6390 and with recombinant V8. It needs to test the antiprotease action of hBD2 in the kinetic reaction where direct interaction of hBD2 with *S.aureus* proteases either neat or present in the supernatant can be measured.

Here is a brief description about the mechanistic insight about the control of *S.aureus* secreted proteases. As described in the first chapter, *S.aureus* pathogenicity depends on the combined action of extracellular toxins, proteases and cell surface proteins. Modulation of virulence expression and synthesis is largely controlled by two global regulatory elements, accessory gene regulator (Agr) and staphylococcal accessory regulator (Sar). Although the production of extracellular proteases varied among clinical isolates of *S.aureus* and their protease production in vitro was primarily determined by the level of *S.aureus* accessory regulator A (SarA) expression, which acts as a repressor **(Anna Karlsson et al., 2002)**. This was supported by inactivation of SarA in three different protease-negative strains which resulted in increased transcription of the

protease genes, while overexpression of sarA in a protease-positive strain completely inhibited protease production (**Arvidson, S et al., 2001).** The alternate transcription factor sigma B is known to affect the expression of several genes encoding virulence factors and stress-response proteins, and seems to counterbalance the influence of the agr system on the expression of virulence factors. The activity of SigB peeks early during the stationary phase of growth **(Senn MM et al., 2005)**. The Agr system and SigB are known to be interconnected **(Bischoff M et al., 2001). (Palma, M., and A. L. Cheung. 2001**). Much of my research aimed at defining the pathogenesis of *S.aureus which had* been done with a limited number of strains, most notably the 8325-4 derivative RN6390. Several lines of evidence indicate that this strain is unique by comparison to clinical isolates of *S. aureus (***Cassat JE et al., 2005)** (**Novick** R.P et al., 2000).

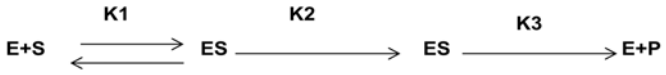
The disturbance of the balance between protease and protease inhibitor could be associated with barrier defects in AD. Another study stated the importance of protease and protease inhibitor balance associated with barrier defect. **Cheng** stated that individuals with AD had decreased expression of cystatin A, which had been found in their epidermal lesions. Reduced inhibition of both endogenous and exogenous cysteine proteases can be associated with barrier defects **(Cheng *et al.*, 2009)**.

A brief description about enzymes and their kinetics is discussed here before starting enzyme kinetics methodology. The enzymes act as catalysts which are not consumed in the reactions. The enzyme produces product at an initial rate that is approximately linear for a short period after that the start of the reaction. As the reaction proceeds and the substrate is consumed, the rate continuously slows. To measure the

initial and maximum rate, enzyme assay are typically carried out while the reaction has progressed only a few percent towards total completion. The length of the initial rate period depends on the assay conditions and can range from milliseconds to hours. Most enzyme studies concentrate on this initial, linear part of enzyme reactions. However it is also possible to measure the complete reaction curve and fit this data to a non-linear rate equation. This way of measuring enzyme reaction is called progress -curve analysis (**Duggleby RG** et al., 1995).

S. aureus secreted proteases act as catalysts which are not consumed in the reactions. The rate of the reaction catalysed by *S. aureus* protease (V8) can be measured by change in the concentration of either substrate or products. The rate of the reaction (v) increases with increasing substrate concentration (S), until proteases become saturated with substrate and the rate of the reaction reaches to Vmax (maximum rate) **(Danson et al., 2002).** A protease inhibitor by definition is a molecule which binds to protease and decreases its activity. The binding of an inhibitor can stop a substrate from entering into the protease active site and/or hinder the protease from catalysing its reaction. The binding of inhibitor with the active site of the protease is either reversible or irreversible **(Rawlings ND et al., 2004)**

Enzyme kinetics was performed by using fluorescence substrate, Z-Phe-Arg-7-amido-4-methyl coumarin. It is generally assumed that serine proteases hydrolysed N-acetyl-amino acid esters and amides through a three-step mechanism involving an acylenzyme intermediate (ES) in addition to the classic Michaelis complex (ES), as given in Equation.



The k1 is an equilibrium constant and K2, k3 are the rate constant of the acylation and deacylation steps. This mechanism can be elucidated by kinetic study by using small synthetic fluorescence substrate. Which allow rapid and precise measurement of *S.aureus* protease activity? The interaction between protease and substrate can cause hydrolysis of the fluorescence substrate, Z-Phe-Arg-7-amido-4-methyl coumarin, by the protease and was measured with a spectrophotometer. It is possible to undertake kinetic studies which might give some information about the mechanism of the *S.aureus* protease-catalysed hydrolysis.

Objective

1-To test the hydrolysis of synthetic substrate with V8 protease kinetic reactions were performed. 2- The enzyme kinetic reactions were performed to explore the proposed, anti-protease action of hBD2, against *S. aureus*, serine proteases.

6.2 Results

Most of the work discussed in the previous chapters performed in-vitro on HaCaT cells monolayer and data was collected where hBD2 indirectly protect the monolayer from *S.aureus* proteases having anti-protease action. In this chapter direct interaction of recombinant and synthetic hBD2 with *S.aureus* proteases either in the supernatant or neat V8 was observed in kinetic reaction**.** In the kinetic assays hydrolysis of the substrate was optimised in a reaction with neat V8 or proteases present in the supernatant. The fluorescence substrate, Z-Phe-Arg-7-amido-4-methyl-coumarin, was hydrolysed by *S.aureus* serine protease during the kinetic reaction and increase in the fluorescence intensity was measured with spectrophotometer. To optimize the enzyme substrate reaction different concentration of substrate were used to achieve a sigmoidal curve. Initially a linear curve was obtained with low substrate concentration. The rate of the reaction (v) increases with increasing substrate concentration (S), until proteases become saturated with substrate and the rate of the reaction reach Vmax (maximum rate). The rate of the reaction was calculated by using following formula $\Delta P/\Delta t=v$ (explain the meaning of v & V)

6.2.1 - Inhibition of the proteases present in the RN supernatant with synthetic hBD2 in a kinetic assay

Protease inhibition activity of proteases present in the RN6390 (derivative of 8325-4 strain) supernatant used with a dilution factor 0.04 (4 µL/100 µL) of the reaction volume here tested. Synthetic hBD2 was added at a concentration of 10 µg/mL and recombinant hBD2 at 1 µg/mL. Synthetic hBD2 was used to reduce substrate hydrolysis by proteases present in the RN6390 supernatant, which resulted in the reduction of fluorescence emission. Substrate hydrolysis by *S. aureus* proteases in the supernatant and their inhibition with 10 µg/mL synthetic and 1 µg/mL recombinant hBD2 was also measured by spectrofluorometry by using the following formula $\Delta P/\Delta t=v$ (Fig 6.1 a). The percentage inhibition of the proteases in the RN6390 supernatant was calculated by comparing it with enzyme substrate progressive curve, which is considered as 100 % acceleration with no inhibition. The ShBD2 has (37 %) reduction of enzyme substrate progressive curve of the protease present in the RN6390 supernatant in the kinetic assay relative to RN6390 which has no inhibition with 100% acceleration. The rhBD2 with concentration of 1 µg/mL attain 33 % inhibition reference to control RN6390 with 100% acceleration and no inhibition (Fig 6.1 b). The rate of the reaction (v) increases with increasing substrate concentration (S), until proteases become saturated with substrate and the rate of the reaction reach Vmax (maximum rate).

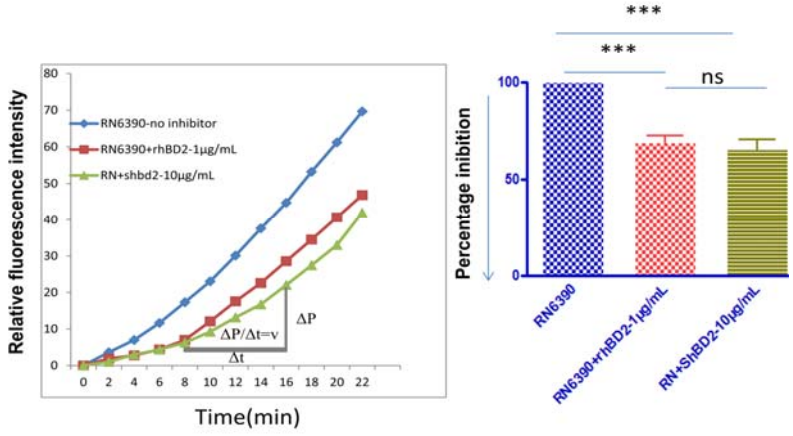


Figure 6.1 Synthetic and r hBD2 action against RN6390 supernatant.

Enzyme kinetic assay was performed (a) 0.04 DF supernatant of RN6390 was used in a kinetic assay with 10 µg/mL synthetic hBD2 (ShBD2) and 1µg/mL of recombinant hBD2 (rhBD2). The change in the relative fluorescent intensity was assayed spectrofluorometrically by using following formula $\Delta P/\Delta t=v$. (b) Data are shown percentage inhibition of the protease in the RN6390 supernatant with 10 µg/mL ShBD2 and 1µg/mL with r hBD2 in reference to control with no inhibition and have 100 % acceleration. Data is representative of n=4 experiments perform with replicates (one way ANOVA).

Tukey's Multiple Comparison Test; (RN6390vsRN6390+ rhBD2-1µg/mL $P < 0.001$) (RN6390vs RN+ShBD2-10µg/mL $P < 0.001$) (RN6390+rhBD2-1mg/mL vs RN+Sbd2-10mg/mL-$P > 0.05$)

6.2.2 - Optimised V8 inhibition with recombinant and synthetic hBD2

Having shown (ShBD2) inhibits V8 protease present in the supernatant of RN6390, I then set out an experiment to look at inhibition of neat V8 protease with the following different forms of hBD2, reduced cysteine amino acid swap with serine hBD2 (C-S hBD2) and scrambled peptide (SCB). Scrambled peptides are a basic, broadly useful type of negative control peptide, useful for testing sequence specificity of the experimental peptide. Since the amino-acid content of control and experimental peptides are identical, which could keep the bulk properties of both control and experimental peptides similar? V8 Protease inhibition by 1 µg/mL recombinant hBD2 (rhBD2), 5µg/mL synthetic cysteine-swap-serine hBD2 (C-ShBD2), and scrambled synthetic peptide (SCB), was assayed spectrofluorometrically. Where recombinant ? and synthetic hBD2 peptides were used to reduce substrate hydrolysis by V8 protease, which result in the reduction of fluorescence emission. 5µg/mL *S. aureus* V8 was pre-incubated for 15 minutes at 37°C with reaction buffer, then the substrate was added. Before measuring fluorescence emission, recombinant and synthetic hBD2 was added separately at concentrations of 500 ng/mL and 5µg/mL, respectively. Hydrolysis of the substrate was reduced by synthetic hBD2, which resulted in the reduction of fluorescence emission. The rate of substrate hydrolysis with V8 protease and their inhibition with 5 µg/mL of synthetic hBD2 was measured by spectrofluorometry by using the following formula $\Delta P/\Delta t=v$. The rate of the reaction was increased by adding substrate until it reached to the maximum velocity of Vmax where I got a hyperbola curve which is considered as a 100 % with no inhibition. I found that 500 ng/mL r hBD2 produced 15 % inhibition (reduce

hydrolysis of the substrate by V8 protease), 5 µg/mL of synthetic peptides have following % age of inhibition, ShBD2 23%, C-S hBD2 18% and SCB peptide 8% respectively relative to 5µg/mL *S. aureus* V8 with 100 % acceleration and no inhibition (Fig 6.2 b).

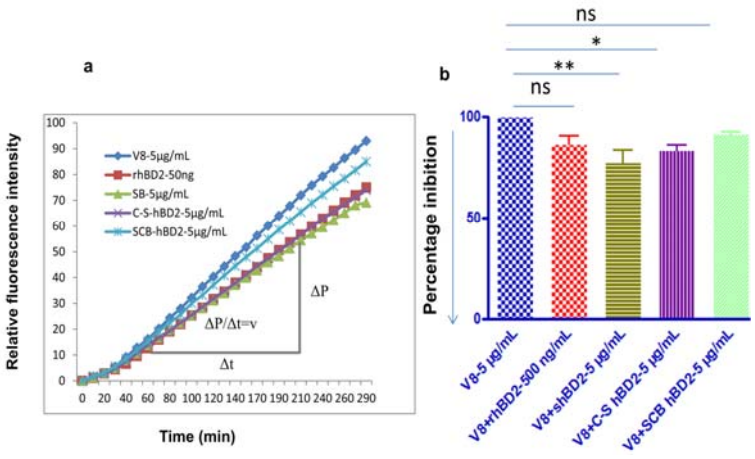


Figure 6.2: kinetic assay with **synthetic** hBD2 **at lower concentrations**

The enzyme kinetic assay was perform to see the effect on V8- substrate acceleration rate with recombinant and synthetic hBD2 peptides (a) The interaction of V8 proteases at concentration 5 µg/mL with 500 ng/mL of recombinant hBD2, 5 µg/mL synthetic hBD2, 5 µg/mL of C-S hBD2 and 5 µg/mL of SCB peptide was assayed spectrofluorometrically by using following formula $\Delta P/\Delta t=v$ (Fig 6.2 a). Data is shown percentage inhibition of the V8 proteases used as control with 500 ng/mL recombinant hBD2, 5µg/mL synthetic hBD2, 5µg/mL Cysteine replace with serine (C-S) hBD2cause and 5µg/mL SCB peptide. Data is representative of n=4 experiments perform with replicate (one way ANOVA)

Tukey's Multiple Comparison Test (V8-5 µg/mL vs V8+rhBD2-500 ng/mL $P > 0.05$) (V8-5 µg/mL vs V8+shBD2-5 µg/mL $P < 0.01$) (V8-5 µg/mL vs V8+C-S hBD2-5 µg/mL $P < 0.05$) (V8-5 µg/mL vs V8+SCB hBD2-5 µg/mL $P > 0.05$) (Fig 6.2 b)

6.2.3 V8 inhibition increased with higher concentrations of synthetic hBD2

Protease inhibition activity of synthetic, cysteine-swap-serine hBD2, and scrambled peptide, at a concentration of 10µg/mL each, was assayed spectrofluorometrically by measuring inhibition of substrate hydrolysis and reduction in the emission of fluorescent intensity by *S. aureus* V8 protease. *S. aureus* V8 at a concentration of 5µg/mL was pre-incubated with reaction buffer for 15 minutes at 37°C, then fluorescent substrate, Z-Phe-Arg-7-amido-4-methylcoumarin, was added. Before measuring the emission of fluorescence, inhibitors were added at the following concentrations: 1µg/mL recombinant and 10µg/mL synthetic, cysteine-swap-serine and scrambled synthetic hBD2. The V8 neat protease with concentration of 5µg/mL interaction with substrate achieve 100 % acceleration which is considered as a control with no inhibition **(Fig 6.3 a)**. The rhBD2 has 15 % inhibition, synthetic hBD2 (ShBD2, C-S hBD2 and SCB) peptide produced inhibition respectively 23%, 18% , and 8% relative to 5µg/mL V8 + substrate reaction with 100 % acceleration, no inhibition **(Fig 6.3 b).** This would state that increasing hBD2 concentration achieve more inhibition of V8 proteases.

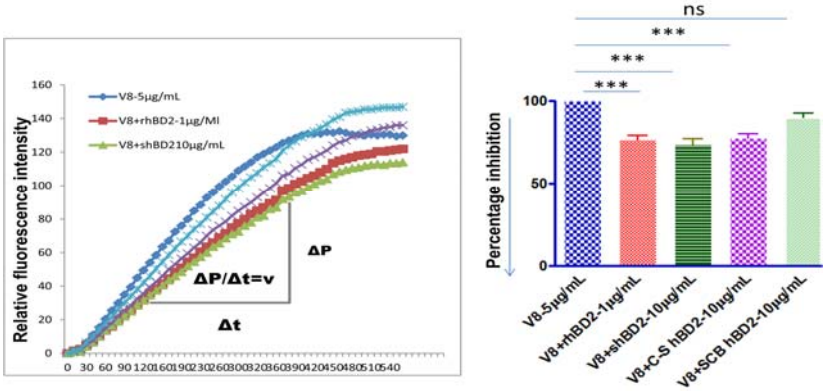


Figure 6.3 V8 kinetic reaction with higher concentrations of Synthetic & rhBD2.

The kinetic assay was perform to see the effect of increasing concentration of recombinant and synthetic hBD2 peptides (a) The interaction of V8 proteases at concentration 5 µg/mL with 1 µg/mL of recombinant hBD2, 10 µg/mL synthetic hBD2, 10 µg/mL of C-S hBD2 and 10 µg/mL of SCB peptide was assayed spectrofluorometrically. Whilst relative fluorescent intensity was measured by using the following formula ΔP/Δt=v. (b) Percentage inhibition of substrate hydrolysis with 1µg/mL of recombinant hBD2, 10µg/mL synthetic hBD2, 10µg/mL with C-S hBD2 and 10µg/mL with SCB peptide. Data is shown as mean fold change (+/- SD) over no inhibitor. Data are representative of n=3 experiments perform with replicate (one way ANOVA). **Tukey's Multiple Comparison Test** (V8-5mg/mL vs V8+rhBD2-1mg/mL $P < 0.001$) (V8-5mg/mL vs V8+shBD2-10mg/mL $P < 0.001$) (V8-5mg/mL vs V8+C-S hBD2-10mg/mL $P < 0.001$) (V8-5mg/mL vs V8+SCB hBD2-10mg/mL $P > 0.05$)

6.3 Discussion-Human serine proteases perform several physiological and cellular functions ranging from degradation and digestive processes to protein processing and tissue remodelling. They share a high degree of amino acid sequence identity in particular they share the histidine-aspartate-serine that is necessary for catalytic activity. Hachem demonstrate the importance of serine protease and their inhibitor balance for normal permeability barrier homeostasis (**Hachem, J.-P et al., 2006).** The presence of a broad range of protease inhibitors in the epidermis could control increase in protease activity due to homeostatic or inflammatory conditions **(Armstrong, P.B., 2006)**. The epidermal protease inhibitors plays role in the maintenance of skin barrier by regulating disturbance of innate immunity caused by external proteases, expressed by several bacterium **(Potempa.J *et al.,* 2009).** The barrier disruption caused by increase serine protease activity could be block by topical serine protease inhibitor application. The protease/ protease inhibitor balance disrupt the natural cycle of cornification (formation of cornified envelop) and desquamation (shedding of the stratum corneum). The barrier abnormalities due to dysfunction of endogenous protease inhibitors were also studied in animals and rodents. A murine model study described a transgenic mouse carrying a null mutation in the gene encoding either cystatin M or cystatin E, died shortly after birth due to severe barrier abnormalities **(Zeeuwen PL *et al.,* 2002)**.

The antiprotease action of hBD2 against *S.aureus* secreted proteases was observed in the kinetic assay where it prevented the cleavage of substrate by *S.aureus* proteases. The hBD2 protected HaCaT cell monolayer from the destructive potential of *S.aureus* secreted proteases by acting as a protease inhibitor. Furthermore, many serine

protease inhibitors, including LEKTI, elafin, SLPI, are believed to control the epidermal serine proteases signaling cascade that can lead to desquamation, or in some cases to inflammation. The relevance of these findings for human disease is underscored by the identification of mutations leading to truncated LEKTI forms in humans with Netherton syndrome. Therefore, fine-tuning of serine protease activity could be an important pharmacological tool in the control of several dermatological diseases **(Leyvraz, C., 2005).** The antiprotease action of hBD2 correlated with SLPI and Elafin which belongs to the Whey acidic protein (WAP) family. The similarity of hBD2 to the members of Whey acidic protein (WAP) family SLPI and Elafin by possessing conserved whey, four disulphide core domain (WFDC). The Whey acidic protein (WAP) family have dual-action, working as an antimicrobial agent and having the ability to inhibit cysteine proteases (**Moreau, T et al., 2008)**. The interaction between protease and substrate was described where hydrolysis of the fluorescence substrate Z-Phe-Arg-7-amido-4-methylcoumarin with *S.aureus* secreted proteases increase in the fluorescence intensity measured with spectrophotometer.

The enzyme kinetics assay was performed to quantify the antiprotease action of recombinant and synthetic hBD2 peptides against *S. aureus*-secreted proteases. The antiprorease effect of recombinant and synthetic hBD2 was observed against both *S.aureus* proteases present in the supernatant and recombinant proteases. I learned from kinetic assay that ShBD2 acts non-specifically against the protease present in the RN6390 supernatant and V8 protease. Increasing the concentration of synthetic ShBD2 peptide provides better inhibition of the protease. Theoretically, by inhibiting *S. aureus*-secreted

proteases which are the external proteases, hBD2 may have another adaptive function to control the rising level of endogenous protease i.e. (metryptase) activity due to homeostatic or inflammatory conditions. Sustained serine proteases activity by prolonged increase in pH leads to degradation of lipid processing enzymes and profound alterations of barrier function and stratum corneum integrity **(Hachem, J.P. et al. 2005)**

The mechanism of antimicrobial action of hBD2 is well-known. It is due to cationic charges, which make electrostatic interactions with the anionic charges on the microbial membrane, which results in dimerization and penetration of the microbial cell wall **(Hoover DM *et al.*, 2000)**. This dimerization is conserved in hBD2, which differentiates it from the other peptides. The hBD2 dimerization allows formation of a higher-order oligomerization of the protein as an octamer. This octamer represents the stable, native, quaternary structure of hBD2. The uniform surface distribution of positive charge residues suggests that hBD2 disrupts the bacterial membrane via electrostatic interactions with the polar head groups of the membrane. It is therefore likely that the octameric that form of hBD2 binds to bacterial membranes (**Hoover DM., 2000)**. The chemical interaction of hBD2 with V8 *S. aureus* serine protease is still not clear.

6.4 Conclusion-The enzyme kinetic study revealed that hBD2, in addition to its antimicrobial action, also protect HaCaT cell from damaging effect of *S.aureus* proteases. I speculate that hBD2, in addition to killing pathogens by invading their membrane, blocks the proteolysis effect of *S. aureus*-secreted proteases. It is quite premature to describe the mechanism of chemical interaction between hBD2 and proteases, which blocks the proteolysis action of *S. aureus* proteases.

Chapter 7: Final discussion:

Atopic dermatitis is a chronic inflammatory disease, 90% of AD patients are *S. aureus* carriers, having the bacteria present in their nasal and perineal areas. Previous studies have confirmed the significance of *S.aureus* proteases in the exacerbation of skin barrier function in atopic dermatitis **(Shaw.L *et al.,* 2004).** *S. aureus* exfoliative toxin (serine protease) is a virulence factor can damage the skin barrier by enzymatic activity having homology to V8 protease **(Amagai *et al.*, 2000).**

As we know tight junctions are dynamically interconnected to the actin cytoskeleton, adherent junctions and desmosomes. There is a structural, as well as a functional; linkage between tight junctions and the actin filament cytoskeleton, via various proteins **(Schneeberger *et al.,* 2004)**. The fate of tight junctions was investigated by exposing HaCaT cell monolayer's to supernatant collected from various *S. aureus* strains.

The deleterious effect of serine and cysteine proteases were analyzed when the monolayer was exposed to recombinant V8 and staphopain B; however staphopain B was unable to damage the HaCaT cell monolayer even at higher concentration. These data suggest that V8 is the most active protease secreted by *S. aureus* into the supernatant and present in the conditioned media used in these studies. It would say, V8 could be the master protease secreted by *S. aureus* involved in deterioration of the skin barrier in individuals with AD. This observation is consistent with another study, demonstrating that extracellular proteases secreted by *S. aureus* cause epidermal barrier dysfunction

(Hirasawa *et al.*, 2009. It is not just the bacterial proteases capable of breaking barrier, common allergens such as house dust mite's secreted serine and cysteine proteases can damage skin barrier **(Chapman et al., 2007).** *S.aureus* may play a role in the chronicity and severity of AD through its release of super-antigenic exotoxin **(Leung et al., 1993).** In addition to their immunological effects these toxins may also directly damage the skin barrier.

The TJ is the main sealing site of the paracellular pathway in epithelia and many endothelia. Tight junctions (TJ) are composed of transmembrane proteins including occludin, the family of claudins and tricellulin. Claudin is known to comprise a multigene family, consisting of more than 20 members **(Morita *et al.*, 1999)**. The two extracellular loops of these proteins link to the extracellular loops from neighbouring cells and by this tighten the Paracellular pathway. Some of the TJ proteins instead of sealing paracellular pathway form paracellular ion channels. *S.aureus* secreted proteases that break down corneodesmosomes by a mechanism similar to that of KLK- (Kallikrein-related epidermal serine proteases) peptidases **(Miedzobrodzki et al., 2002).**

Morphological and physiological studies revealed that tight junctions are not a simple barrier. Tight junctions are ion-and size-selective and their barrier function varies significantly in tightness, depending on the cell type and physiological requirement **(Diamond JM *et al.*, 1977).** A murine model study described how Claudin (CLDN-1) and E-cadherin knock-out mice can cause deficiency of tight junction proteins which characterised by a leaky barrier. The affected mice died soon after birth due to tremendous transepidermal water loss **(Furuse *et al.*, 2002)**. The functional importance of

tight junction proteins for the maintenance of the skin barrier against outside-in entrance of the environmental allergens was examined. The effect of *S. aureus*-secreted proteases in the dissolution of the tight junction protein Claudin-1 and ZO-1 was studied and quantified. ZO family proteins are the cross–linkers which anchor TJ proteins to the actin cytoskeleton. Damage to the TJ may cause dissolution of the actin cytoskeleton, which can be seen by staining HaCaT cells with Phalloidin-FITC.

S.aureus V8 protease cause damage to the monolayer results in the loss of cells at that area therefore no cells means reduction in the CLDN-1 staining at the damaged area **(Fig 5.5 b-c)**. I presume epidermal barrier in AD which correlate with HaCaT cell monolayer and damage to claudin-1 caused by *S. aureus* proteases can disrupt the cell-cell interactions, of HaCaT cells monolayer. Tight junction's barrier function varies significantly in tightness, depending on the cell type and physiological requirement (**Diamond *et al.*, 1977**). **(Furuse, M et al., 2002).** Damage to Claudin-1 by *S. aureus*-secreted proteases was quantified by Western Blot assay, where the reduction of Claudin band intensity was observed.

The ion permeability of the paracellular pathway could be the pathogenesis of inflammatory or infectious diseases turned out to cause, or to be caused by, paracellular barrier defects (**Amasheh, S et al., 2002**). The **Brandner** previously described mechanism of regulating tight junctions against *S. aureus*-secreted proteases. (**Brandner JM et al., 2009).** They demonstrated that barriers for diverse ions and molecules of different size are formed in keratinocytes by TJs, and TJ proteins CLDN 1, CLDN 4, OCLN, and ZO1. TJs also form a barrier for water, but CLDN 1 and CLDN 4 are

dispensable for this barrier, however CLDN 1 influences important component of the stratum corneum (SC) barrier.

The skin epidermal barrier can be protected by stimulating with secretery LTA released by *S. epidermidis* and Vitamin D. Part of skin defense against *S. aureus* exoproteases can be maintained by *S. epidermidis* a skin commensal by releasing Lipid S' secretary LTA, a cell wall component, into the medium during growth **(Schroder *et al.*, 2003).** The role played by skin commensal against the pathogen was observed when heat killed *S.epidermidis* (HKSE)-stimulated HaCaT cells were protected from the deleterious effect of the proteases present in the supernatant of *S.aureus* 8325-4 and V8 protease. While unstimulated HaCaT cells were not protected from the proteases present in the supernatant of *S.aureus* 8325-4 and V8 protease.

Internal defense system of epidermis was also tested when HaCaT cells were stimulated with Vitamin D3 and then exposed with the proteases present in the supernatant of *S.aureus* 8325-4 and V8 protease. The Vitamin D3 stimulated HaCaT cells were protected from the effect of the proteases and upregulation of hBD2 was assessed by performing ELISA. Vitamin D3 is another external factor; stimulate epidermal defence by up regulating AMP. 1, 25 OH D3 regulates keratinocyte prolifiration, differentiation and the formation of intact epidermal barrier. Alteration in local vitamin D3 concentration and or activation will likely affect normal cutaneous immune function, barrier function and inflammation **(Bikle DD, *et al*, 2004)**. Data presented in chapter 4 described how Vitamin D-stimulated HaCaT cells protected HaCaT cells monolayer from *S.aureus* secreted proteases. Importance of Vitamin D has been observed when

inflammation in individuals with AD was reduced with UVB radiation. In normal conditions, keratinocytes express very low quantities of AMP, but during infection, inflammation and wounding its expression is up-regulated and AMPs accumulate in the lamellar bodies of the SG of the epidermis **(Gallo *et al.*, 2002)**.

I then aimed to establish whether the presence of Vitamin D3 (which is protective in atopic dermatitis) could also have a protective effect. In these studies HaCaT cells monolayer protection with vitamin D3 was assessed by visualization of the monolayer and by measuring TEER. I, observe TEER of the monolayer is inversely proportion to the TJ permeability. Hence, as TJ becomes less permeable, the TEER is increased. Conversely, as permeability goes up, TEER is reduced. The use of HaCaT cells monolayer offers the possibility of measuring TJ function in response to *S. aureus* proteases in a simple assay.

There was significant drop in the TEER reading in unstimulated monolayer when exposed to V8 protease for 24 hour **(Fig 4.3 b).** In contrast, 1, 25 OH Vitamin D3 -pre-stimulated HaCaT monolayer's, TEER was increased and there was no damage to the monolayer after 24 hours of V8 exposure **(Fig 4.3 c).** In order to test that hBD2 was the key protective factor released in response to Vitamin D3, HKSE and IL1-β/LTA stimulation, ELISA was performed. I found encouraging results with ELISA which stated that hBD2 secretion increased by stimulating HaCaT cells with Vitamin D3, HKSE and IL1-β/LTA.

As disruption of the monolayer by *S.aureus* proteases, can be prevented by blocking the interaction of protease with peptide, which become substrate for the proteases. I presume epidermal barrier in AD which correlate with HaCaT cell monolayer can also be protected from *S.aureus* proteases. The leekage of tight junctions permeability barrier was investigated by exposing HaCaT cell monolayers to supernatant collected from various *S. aureus* strains, and blocked their effect with prior treatment with synthetic hBD2 or exposing hBD2 stable cell line with protease. Damage to Claudin-1 by *S. aureus*-secreted proteases, and their protection by hBD2, was quantified by Western Blot assay. Two different techniques were used to test the anti-protease effect of hBD2. IL1-β/LTA externally stimulated HaCaT cells and internally hBD2 over-expressing stable HaCaT cell line were exposed to recombinant V8 and protease present in the supernatant of 8325-4. The hBD2 over-expressing stable HaCaT cells line was compared by adding synthetic hBD2, before exposing them to V8 and proteases present in the supernatant. I found synthetic hBD2 and hBD2 over-express HaCaT cell line protect the monolayer against 4µg/mL of V8 but unable to protect 8µg/mL of V8. The indirect antiprotease action of hBD2 was observed when IL1-β/LTA and Vitamin D3 stimulated HaCaT cells were protected from *S.auerus* secreted proteases. These observation suggest that hBD2, in addition to killing pathogens by invading their membranes, blocks the proteolytic effect of *S. aureus*-secreted proteases.

To assess, directly, the link between hBD2 expression and permeability barrier function, I restored permeability barrier homeostasis artificially, by growing HaCaT cells on a transwell membrane. The artificial barrier was disturbed by exposing it to 4 and 8

µg/mL of V8 protease for 24 hours. In order to prevent this alteration in permeability barrier I grew normal HaCaT cells in different wells. Normal HaCaT cells were pre-treated with 4 µg/mL of synthetic hBD2 before exposed them with 4 and 8 µg/mL of V8 protease.

Next, 1 restored permeability barrier homeostasis, naturally, by growing hBD2 over-expressing HaCaT cell-lines on the transwell. The hBD2 over-expressing HaCaT cell-lines monolayer exposed with 4 and 8 µg/mL of V8 protease for 24 hours. Artificial permeability barrier homeostasis was quantified by using FITC-alb fluorescence dye. FITC-alb dye transportation was facilitated across the gaps produced between the cells due to damage caused by 4 and 8 µg/mL of V8 protease. The FITC-labelled albumin model was preferred to elucidate the importance of skin barrier against outside-in entrance of the environmental allergens in AD. FITC-labelled albumin has been used as a substrate for the proteases and also has been used to detect sites of protein leakage in the lungs **(K. Matter and M. S. Balda, 2003)**. FITC-labelled albumin Penetration through the intercellular junction, correlate with leaky barrier in AD, which cause outside-in entrance of environmental allergens **(Schneeberger *et al.*, 2004)**. The rate of FITC-alb dye transportation across the disturbed barrier was quantitated by measuring increase in the fluorescence intensity with spectrophotometer after 8 hours **(Fig 4.4 a).**

As patients with Atopic dermatitis (AD) are more prone to *S.aureus* infections which could be due to low hBD2 copy number. A study was done by Gambichler. T in 2008, which stated that most antimicrobial peptides (AMP's) investigated in that study proved to be over expressed in atopic patients as well as Psoriatic patients when

compared to control. However, a statistically significant difference in AMP mRNA expression between atopics and Psoriatic patients was only found for hBD2. **(Gmbichler et al., 2008)**. In another study it stated that the DNA copy number of the beta defensin gene cluster on chromosome 8q23 was highly polymorphic within the healthy population. It is suggested that defective hBD2 induction in crohn disease (CD) patients could be due to low DEFB4 gene (hBD2) copy number. I aimed to investigate mRNA expression level of hBD2 in human keratinocyte cell line HaCaT cells and then compare it with 18S ribosomal RNA as a control. For this purpose QRT-PCR was performed to find about the expression levels of hBD2 in both stimulated and unstimulated cells. 18S rRNA is a component of the small eukaryotic ribosomal submit (40S) and structural RNA for the small component of ribosome's and thus one of the basic components of all eukaryotic cells. The genes coding for 18S rRNA are referred to as 18S rDNA. Sequence data from this gene is widely used in molecular analysis to reconstruct the evolutionary history of organism. The 18S rRNA gene is one of the most frequently used genes in polygenetic studies and an important marker for random target PCR in environmental biodiversity screening. In general rRNA gene sequences are easy to access due to highly conserved flanking regions allowing for the use of universal primers (**Meyer A et al., 2010).** Their repetitive arrangement within the genome provide excessive amount of template DNA for PCR, 18S gene is part of the ribosomal functional core. QRT-PCR was performed to find out that *DEFB4* gene expression either in over-expressing stable cell line or cells stimulated with IL1-β/LTA. The QRT-PCR results showed upregulation of DEFB4 in wild type HaCaT cell stimulated with 100ng IL1-β and hBD2 stable cell line. The

DEFB4 mRNA expression level when normalised to 18s was 1000 fold with IL1-β and 1200 fold with hBD2 over-expressing stable line, which was higher than the WT.

To confirm that protection is due to increase secretion of hBD2 when stimulated with Vitamin D3, HKSE and IL1-β/LTA, we knocked down hBD2 in HaCaT cells with siRNA. We quantified our results by calculating percentage damaged to the monolayer caused by the protease. Not surprisingly, cells that had not been stimulated to make hBD2 had a similar phenotype whether they expressed the siRNA or not. We observed that *DEFB4*-siRNA knock-down HaCaT cells blocked the protection afforded by IL1-β/LTA stimulation, whilst control-siRNA knock-down HaCaT cells cannot block the projection afforded by IL1-β/LTA stimulation. In order to clearly assess the impact of hBD2 expression by HaCaT cells, I assessed the responses of stable hBD2 over-expressing HaCaT cell lines. Would they be protected from the *S. aureus*-secreted proteases? I found that, hBD2 over-expressing HaCaT cell lines protected from the damaging effect of V8 proteases.

Hoover described that antimicrobial action of hBD2 is due to cationic charges which make electrostatic interactions with the anionic charges on the microbial membrane, results in dimerization and penetration of the microbial cell wall **(Hoover DM *et al.*, 2000)**.The direct interaction of the hBD2 with *S.auerus* secreted proteases was studied by enzyme kinetics. It was demonstrated that hBD2; in addition to its antimicrobial action have antiprotease action by having direct interaction with protease. The enzyme kinetics assay confirmed that increasing hBD2 concentration has more profound effect in the inhibition of V8 protease **(Fig 6.3).**

7.1 **Conclusion**

It was found that *DEFB4* (hBD2) *knock*-out cells were unable to block *S. aureus*-secreted proteases, whilst hBD2 over-expressing HaCaT cells were protected. The anti-protease effect of hBD2 was also tested by inducing hBD2, with IL1-β/LTA, Vitamin D3 and HKSE Stimulated HaCaT cells, which protects them from *S. aureus*-secreted proteases. AMPs are known to be multi-factorial having a role as a mediator of inflammation effects on epithelial and inflammatory cells. AMP also has the ability to modulate inflammation and immunity by functioning as immunomodulators **(Hunter *et al.*, 2002).** AMP has been demonstrated to kill gram+ve and gram-ve bacteria's, enveloped viruses, fungi and even cancerous cells **(Hoskin *et al.*, 2008).** The anti-protease effect of hBD2 was also evaluated in the enzyme kinetics assay. After observing all the quantitative results, we can propose a novel anti-protease effect of hBD2. This study is the first to show that hBD2, in addition to killing pathogens by invading their membrane, blocks the proteolytic effect of *S. aureus*-secreted proteases.

7.2 Future plans-After this study it needs to be determined the chemical mechanism of protease inhibition? Which could be either direct binding to the active site of the protease, or hydrogen bonding with the enzyme-substrate complex? With the received information, we aim for further optimization of the inhibitor in respect of activity, selectivity and toxicity. Further study will focus on the effects of environmental and skin condition as well as different pH values. We have future plan to determine the (Ki) value of hBD2 for V8, and staphopain B. NMR-spectroscopic study, co-crystallization and structure determination of enzyme- inhibitor complex will determine.

7.3 General conclusion and future work

7.3.1 Novel finding

1- The hBD2 can protect HaCaT cell monolayer by the *S.aureus* secreted protease as an inhibitors

2- Vitamin D protect the monolayer by expressing hBD2

3- V8 is the most active protease secreted in the CM

4- TJ protein Claudin-1 become a substrate for V8 protease

5- The hBD2 over-express HaCaT cell stable lines can be established

7.3.2 Strength and weaknesses

The major strength of this work is that it has been done in skin cell line hence the results of my study are highly relevant to skin biology. -The major weakness of this study is the lack of functional data in human skin. Ultimately understanding how *S.aureus* proteases disturb epidermal barrier by breaking tight junction (TJ) protein. how hBD2 can protect the barrier by blocking *S.aureus* proteases by having antiproteases effect- Selection ofthe proper application, by which hBD2 can help in the repair of skin barrier.

7.4 References

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7.7- Appendices

7.7A preparation of Immunohistochemistry solutions

Immunohistochemistry solutions	Preparation
4%(v/v) Paraformaldehyde	A mixture of 1 phosphate buffer saline tablet and 8g paraformaldehyde (PFA) in 200 mL distilled water was stirred and heated for 20 minutes until the PFA was dissolved.
1x phosphate buffer saline (PBS)	(0.137M NaCl, 0.05 M NaH2PO, pH 7.4
o.5 % (v/v) Triton-X 100	A mixture of 500 µL Triton X-100 and 100mL 1X TBS.
Blocking solutions 1% blocking solution: 3% blocking solution:	50 µL sera and 0.05g bovine serum albumin (BSA) were added to 5mL 1X TBS 150 µL sere and 0.1g bovine serum albumin were added to 5mL 1X TBS

7.7 B preparation of Western blotting acrylamide gel

	4.5%Gel	6%	9%	12%	15%	16.5%
Protoflow acrylamide gel	1.5 mL	2 mL	3 mL	4 mL	5 mL	5.5 mL
2M-Tris-pH 8.8	2 mL	2 mL	2 mL	2 mL	2 mL	2 mL
H2O	6.5 mL	6 mL	5 mL	4 mL	3 mL	2.5 mL
10%SDS	150µL	150µL	150µL	150µL	150µL	150µL
TEMED	15 µL	15 µL	15 µL	15 µL	15 µL	15 µL
10% APS	150µL	150µL	150µL	150µL	150µL	150µL

7.7 C preparing stacking gel

Protoflow acrylamide gel	700 µL
H20	600 µL
0.5M Tris pH 6.8	600 µL
H2O	3mL
10% SDS	50 µL
TEMED	5 µL
10%APS	50 µL

7.7 D Western blotting solutions

Western blotting solutions	Preparation
- APS 10%	1g Ammonium per sulphate in 10 mL of distilled water
Blocking Buffer	5% (w/v) dried skim milk in PBS+0.05%Tween 20
Running Buffer 1.5M PH 8.8	90.75 g Tris in 500 mL of distilled water adjust the pH 8.8 with HCL
SDS 10% W/V	100g of Sodium Dodecyl Sulphate in 1000 mL of distilled water
Stacking Buffer 0.5 M, PH 6.8	6g Tris in 100g distilled water adjust the PH at 6.8 with HCL
Tank buffer 10x	72g glycine 50 mL of SDS complete to 500 mL with distilled water
TBS I0X	24.2 g Tris and 84 g NaCl adjust the PH to 7.6 with HCL complete to 1L with distilled water
Transfer Buffer 1X	3.03 g Tris HCL 14.4 g glycine 200 mL methenal 1mL SDS complete to with 1L of distilled water

7.7 E Measurement of Trans-Epithelial Electrical Resistance (TEER) (Millipore)

1	Electrodes were sterilized in 70% ethanol for 15 mint and then kept in the medium used to culture the cells
2	Keep the shorter tip of electrodes in the culture plate insert in such a way that it should not contact cells grown on the membrane.
3	The longer tip should be placed in the outer well and it should just touch the bottom of the outer well.
4	To get stable and reproducible results, the electrodes are held steady and at a 90° angle to the plate insert.
5	First measure the resistance across the blank insert with no cells
6	Each insert was measured at 3 different sites and the mean values were calculated. Unit area = cm2